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Prostate Cancer

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13. ABSTRACT (Maximum 200 Words) The goal of this proposal was to evaluate prostatic acid phosphatase (PAP), a prostate tissue-specific protein, as a candidate tumor antigen for a prostate cancer vaccine. The specific aims of the current proposal were: (1) to determine whether patients with prostate cancer have a pre-existing CD4+ T cell immunity to PAP, and (2) to determine whether patients with prostate cancer have a pre-existing CD8+ T cell responses to PAP and whether PAP-specific CTL derived from individual patients can lyse prostate tumor cells. Results from the studies demonstrate that patients with prostate cancer have detectable Th responses specific for PAP, typically of a Th1 phenotype, implying an immune environment capable of supporting an inflammatory/CTL response specific for PAP, and further suggesting the concept of vaccine strategies targeting PAP, because tolerance to this "self" protein can be overcome <i>in vivo</i> . In addition, 2 Th epitopes capable of eliciting PAP-specific Th responses <i>in vitro</i> were identified, and 3 HLA-A2 epitopes specific for PAP were identified. The identification of these epitopes has laid the foundation for future clinical peptide-based vaccine trials with the goal of eliciting PAP-specific Th and CTL responses.				
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Final Report for Proposal: *Evaluation of Prostatic Acid Phosphatase (PAP) as a Candidate Antigen for the Development of Cancer Vaccines for Prostate Cancer.*

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**INTRODUCTION:** The goal of this research has been to evaluate prostatic acid phosphatase (PAP), a prostate tissue-specific protein, as a candidate tumor antigen for a prostate cancer vaccine. As stated in the original proposal and statement of work, the specific aims of the work were to: (1) determine whether patients with prostate cancer have a pre-existing CD4+ T cell immunity to PAP, and (2) determine whether patients with prostate cancer have a pre-existing CD8+ T cell responses to PAP and whether PAP-specific CTL derived from individual patients can lyse prostate tumor cells. This final report will review the accomplishments made over the two years of funding with respect to the specific aims and statement of work of the project, in particular over the last year of funding.

**Aim 1: To determine whether patients with prostate cancer have pre-existing CD4+ T cell immunity to PAP.**

□ **Purification of PAP from CHO cells – TASK FINISHED**

As detailed in the first yearly report, during the first three months of work I was unsuccessful in generating sufficient quantities of purified recombinant human PAP from CHO cells to perform the T cell work proposed. Therefore, the studies below were initiated and completed using highly purified human PAP available through commercial vendors (Research Diagnostics, Inc. and Chemicon International).

□ **Collection of blood and proliferative T cell assays from 120 patients and 30 normal patients – TASK FINISHED**

As described in the first yearly report, the initial scope of this task was scaled down to an analysis of 80 patients with varying stages of disease (20 patients per group as originally described) and 20 volunteer control donors. The work was extended, however, to also look at T cell proliferative responses to PSA in the same patient panel. The completed analysis demonstrated that 14% (11/80) of patients have significant (stimulation index > 2) CD4+ T helper cell responses to PAP. This is not statistically different from the control population, in which 5% (1/20) controls also had a detectable T cell response to PAP, and no differences were noted among the subpopulations of patients. These results were in contrast to those found for PSA, in which 7.5% of patients had significant responses to PSA compared with none of the controls. Responses were also more common in patients with metastatic disease (15%, 6/40) compared with early stage disease (0%, 0/40). These results were presented in a poster presentation at the April 2000 national meeting of the American Association for Cancer Research, and were accepted for publication in *Prostate* (see Appendix).

□ **Characterization of cytokine profile for PAP-specific helper T cell responses – TASK FINISHED**

The CD4+ helper T cell responses to both PAP and PSA above were characterized by cytokine release and shown to be predominantly IFN $\gamma$ -secreting as opposed to IL-5-secreting, consistent with a Th1-like response. Taken together, these results have been significant to demonstrate that patients with prostate cancer do indeed have low-level T cell responses to PAP, and the type of T cell response is consistent with a potentially therapeutic response. These results were presented in the same poster presentation as above at the April 2000 national meeting of the American Association for Cancer Research, and were reported in the publication noted above (see Appendix).

□ **Characterization of antibody responses to PAP in 120 patients and 100 normal controls – TASK FINISHED**

As described in the first yearly report, the analysis proposed was extended to a larger patient population (200 patients) and to a larger analysis of other prostate cancer-associated proteins. Antibody responses were consequently analyzed by ELISA to PAP, PSA, p53 and HER-2/neu in the same population of 200 patients. Only a minority of patients (6%, 12/200) had a detectable antibody titer to PAP, and this was not statistically distinct from the control population (7%, 7/100,  $p=0.74$ ). Similar results were found for p53. Antibody responses were significantly different in the patient populations for PSA (11%, 22/200) and HER-2/neu (15.5%, 31/200) compared with controls (3%, 3/100,  $p=0.02$ , and 2%, 2/100,  $p=0.0004$  respectively). These responses were most prevalent in the subpopulation of patients with androgen-independent prostate cancer. These results have suggested that the primary immune response to PAP is cellular, and Th1-biased, not antibody-biased. This antibody study in particular, however, has demonstrated that patients with prostate cancer, and even late-stage patients, are able to mount an

antigen-specific immune response to proteins made by their tumors. These results were published in the *Journal of Urology* (see Appendix).

□ Characterization of T helper cell peptide epitopes recognized – TASK COMPLETED

The immunogenicity of PAP-specific T helper cell epitopes has been evaluated in 20 male subjects with (15) and without (5) prostate cancer. While the initial proposal was to evaluate 40 patients overall, 20 patients with proliferative responses to PAP, and 20 without, this was not feasible given the low frequency of responses found above and the availability of cryopreserved specimens for the analysis. Consequently, T cell responses to individual peptides have been analyzed in 7 patients with T cell responses to PAP and 13 patients without T cell responses to PAP. These studies identified 4 peptides that are likely CD4+ T cell epitopes, in that they were recognized only in patients with T cell responses to PAP. These results were presented at a poster presentation at the American Association for Cancer Research annual meeting in April 2000. In the interim since the last yearly report, short term T cell lines specific for these 4 peptides were generated using PBMC from patients with prostate cancer and without T cell responses to PAP. Peptide-specific lines from 2 of the 4 peptides were found to proliferate in response to PAP, implicating these peptides as naturally processed Th epitopes. These studies suggest that these epitopes may be useful for inclusion in vaccines targeting PAP with the goal of eliciting Th responses specific for PAP, and have laid the foundation for future peptide-based clinical vaccine trials targeting PAP. These results were submitted and accepted for publication in *Cancer Research* (see Appendix).

**Aim 2: To determine whether patients with prostate cancer have a pre-existing CD8+ T cell responses to PAP and whether PAP-specific CTL derived from individual patients can lyse prostate tumor cells.**

□ Generation of target cell lines from subject fibroblasts and B-LCL – TASK NEARLY COMPLETE

To date, we have established fibroblast lines and EBV-transformed B cell lines in the context of other funded clinical studies in 20 HLA-A2 expressing patients with prostate cancer. These cell lines will be used as stimulator and target cell lines for experiments related to those proposed. A retroviral construct (pLNCX) containing the cDNA for human PAP has been used to transform these cell lines to express PAP. As described below, these lines will be used as target cells for cytotoxicity testing of peptide-specific lines only.

□ Determination of PAP-specific CTL frequency by limiting dilution in a chromium release assay for 10 normals and 20 patients with prostate cancer – TASK DELETED

Pilot experiments using autologous stimulator cells from multiple patients expressing PAP did not show any PAP-specific CTL activity after multiple *in vitro* stimulations. This approach was felt to not be feasible, and consequently this task, and the similar task of evaluation by ELISPOT were deleted.

□ Determination of PAP-specific CTL frequency by ELISPOT for 10 normals and 20 patients with prostate cancer – TASK DELETED (see above)

□ Define HLA-A2 epitopes able to generate PAP-specific CTL using patient PBMC *in vitro* – TASK COMPLETED – ONGOING RELATED WORK

As described in the previous yearly report, eleven potential HLA-A2 binding peptide epitopes from PAP were constructed and, in a project not identified in the proposal, were ranked in an *in vitro* T2 binding assay for their true binding affinity for HLA-A2. These studies identified 6 peptides with moderate-to-high binding affinity to HLA-A2. These peptides were then used as stimulator antigens to generate peptide-specific T cell lines from PBMC obtained from ten HLA-

A2 expressing patients. After multiple *in vitro* stimulations I was unable to detect reproducible peptide-specific CTL activity as determined by chromium release assay using peptide-loaded target cells. This approach was therefore abandoned. However, PBMC from 20 HLA-A2 expressing individuals and 10 HLA-A2 expressing males without prostate cancer were used to evaluate peptide-specific responses with these 11 peptides as defined by IFN $\gamma$  release in response to peptide stimulation in an ELISPOT assay. This method identified 3 peptides for which both patients and controls had high peptide-specific T cell frequencies, implicating these as MHC class I epitopes. These were peptides found to have moderate-high specificity for HLA-A2 by *in vitro* T2 assays, and had not been previously identified as PAP epitopes. This work received a 2001 AFLCAC Scholar in Training Award and was presented in a poster at the 2001 national meeting of the American Association for Cancer Research. Work is ongoing to culture T cell lines on these specific peptides using PBMC from patients with the highest T cell frequencies as identified by ELISPOT, to confirm that peptide-specific T cells exhibit cytolytic activity. At that point, these results will be reported as a manuscript. In addition, the target cells generated from the task above from autologous systems and engineered to express PAP will be used to confirm that T cell lines exhibit cytolytic activity against autologous cells expressing PAP. While this is not the work initially proposed, this has turned out to be a more exciting and fruitful avenue of investigation.

- Define HLA-A2 epitopes able to generate PAP-specific CTL using HLA-A2 transgenic mouse model *in vivo* system – TASK COMPLETE

As described in the previous annual report, pilot experiments revealed that simple immunization with a boost 2 weeks later were not sufficient to initiate a peptide-specific T cell response. Other pilot experiments using well-characterized HLA-A2 binding peptides (influenza matrix peptide, EBV 9-mer HLA-A2 epitope) with and without helper T cell epitopes likewise gave poor and inconsistent results. This approach was therefore abandoned, particularly since direct assay of PBMC from patients with prostate cancer (as described above) identified HLA-A2 epitopes directly, without the need for an animal model.

- Determine whether T cell lines and clones specific for PAP-derived CTL peptides can lyse autologous cells expressing PAP and/or HLA-matched prostate tumor cell lines – TASK COMPLETE – RELATED EXPERIMENTS ONGOING

In pilot assays, all 11 peptides were used as stimulator antigens to attempt to generate peptide-specific T cell lines in ten HLA-A2 expressing patients. After multiple *in vitro* stimulations I was unable to detect reproducible peptide-specific CTL activity as determined by chromium release assay using peptide-loaded target cells, let alone autologous targets expressing PAP or prostate tumor cell lines. This approach, therefore, was abandoned as not feasible. However, as described above, related work is ongoing following the identification of 3 specific HLA-A2 epitopes for PAP found by the ELISPOT. In this work, T cell lines are being cultured on specific peptides using PBMC from patients with the highest T cell frequencies identified by ELISPOT. These lines will then be tested for cytolytic activity using peptide-pulsed HLA-A2 expressing target cells, autologous cells expressing PAP, and the LNCaP HLA-A2-expressing prostate cancer cell line.

### **Key Research Accomplishments:**

- ❑ Identification of helper T cell responses, predominantly of Th1 type, in patients with prostate cancer specific for PAP. This suggests that a potentially therapeutic immune response to PAP can exist *in vivo* and therefore may either be initiated or augmented by means of antigen-specific vaccination.
- ❑ Evaluation of humoral immune responses to several prostate cancer-associated antigens has demonstrated that antibody responses to PAP are rare compared with PSA or HER-2/neu, and that the majority of pre-existent immune responses to PAP are cellular responses.
- ❑ Identification of 2 potential CD4 T cell epitopes that may be useful in clinical trials to elicit helper T cell responses to PAP.
- ❑ Identification of 3 peptides derived from the amino acid sequence of PAP which exhibit moderate-to-high binding to HLA-A2 in a T2 *in vitro* binding assay, and which are recognized by peptide-specific T cells in patients with prostate cancer, suggesting these may be useful HLA-A2-restricted CD8 T cell epitopes for use in clinical trials.



## Reportable Outcomes:

- ❑ Manuscripts, abstracts and presentations:
  - ❑ **McNeel DG, Vessella R, Lange PH, and Disis ML.** (1999) "Antibody immunity to prostate cancer antigens can be detected in patients with prostate cancer." *Proc. Amer. Assn. Cancer Res.* **40**:353.
  - ❑ **McNeel DG, Nguyen LD, Disis ML** (2000) "T cells derived from patients with prostate cancer secrete IFN $\gamma$  in response to stimulation with prostate cancer antigens." *Proc. Amer. Assn. Cancer Res.* **41**:880.
  - ❑ **McNeel DG, Nguyen LD, Disis ML** (2000) "Identification of potential CD4+ T helper epitopes derived from the protein sequence of prostatic acid phosphatase (PAP)." *Proc. Amer. Assn. Cancer Res.* **41**:699.
  - ❑ **McNeel DG, Nguyen LD, Storer BE, Vessella R, Lange PH, and Disis ML.** (2000) "Antibody immunity to prostate cancer-associated antigens can be detected in the serum of patients with prostate cancer." *J. Urol.* **164**:1825-1829. (see Appendix)
  - ❑ **McNeel DG, Disis ML.** (2000) "Tumor vaccines for the management of prostate cancer." *Arch. Immunol. Ther. Exp.* **48**:85-93. (see Appendix)
  - ❑ **McNeel DG, Knutson KL, Disis ML** (2001) "Identification of PAP-specific MHC class I peptide epitopes by screening patients with prostate cancer by IFN $\gamma$  ELISPOT." *Proc. Amer. Assn. Cancer Res.* **42**:277.
  - ❑ **McNeel DG, Nguyen LD, Disis ML.** (2001) "Identification of T helper epitopes derived from prostatic acid phosphatase." *Cancer Res.* **61**:5161-5167. (see Appendix)
  - ❑ **McNeel DG, Nguyen LD, Ellis WJ, Higano CS, Lange PH, Disis ML.** (2001) "Naturally occurring prostate cancer antigen-specific T cell responses of a Th1 phenotype can be detected in patients with prostate cancer." *Prostate* **47**:222-229. (see Appendix)
- ❑ Patents and licenses – NONE
- ❑ Degrees obtained – NONE
- ❑ Development of cell lines, tissue or serum repositories:

Cryopreserved fibroblast lines and peripheral blood mononuclear cells obtained from other clinical studies have been used to establish autologous systems with fibroblast lines and EBV-transformed B cell lines from individual patients. Work is ongoing to establish PAP-expressing fibroblast lines and B-LCL lines. Likewise, archived serum samples obtained from other clinical studies was used to accomplish the work outlined in Aim 1 above.
- ❑ Informatics – NONE
- ❑ Funding applied for based on work supported by this award:

An application for a National Institutes of Health K23 (Mentored physician scientist award) is pending, the aims of which follow directly from the research funded by this DOD Award. The aims proposed in the new proposal are to 1) identify the lymphocyte populations responsible for eliciting and maintaining prostatitis in a rat model using a DNA-based vaccine targeting PAP; 2) identify serologic antigens in patients with prostatitis that are recognized in patients with prostate cancer; and 3) determine if PAP-specific CD8+ T cells can be elicited in patients with prostate cancer by means of a DNA-based vaccine in the context of a clinical trial.
- ❑ Employment or research opportunities applied for:

I accepted a position as Assistant Professor of Medicine within the Experimental Therapeutics group of the Section of Medical Oncology in the Department of Medicine at the University of

Wisconsin, Madison, on 7/1/01. Duties there will involve 20% clinical activities (caring for patients with genitourinary malignancies) and 80% time devoted to research (immunological treatment of prostate cancer). I am indeed grateful for the period of research support provided by the Department of Defense to allow me to bridge to this independent position in translational clinical research of prostate cancer. The support provided is truly critical for early investigators, and has allowed me to stay within the important field of prostate cancer research.

- Personnel receiving pay from research effort:
  - Lan D. Nguyen, research technologist I
  - Sarah Pelonio, administrative student helper

**Conclusions:**

The goal of this research has been to evaluate prostatic acid phosphatase (PAP), a prostate tissue-specific protein, as a candidate tumor antigen for a prostate cancer vaccine. As a result of this project, it has been demonstrated that patients with prostate cancer have existing immunological responses specific for PAP, implying that vaccine strategies may be feasible to overcome immunological tolerance to this "self" protein. Whereas antibody responses specific for PAP were rare among patients, Th responses specific for PAP could be detected, and these tended to be of a Th1 phenotype, implying an immunological environment capable of supporting an inflammatory/CTL response specific for PAP. In addition, 2 Th epitopes capable of eliciting PAP-specific Th responses *in vitro* were identified, and 3 HLA-A2 epitopes from PAP were identified using PBMC from patients with prostate cancer. The identification of these epitopes has laid the foundation for a clinical vaccine trial using PAP-specific peptides with the goal of eliciting PAP-specific Th and CTL responses. In addition, work funded elsewhere has identified nucleic acid vaccines targeting PAP as an effective strategy in animal models to elicit PAP-specific CTL and destructive prostatitis. Together, these experiments have succeeded in identifying PAP as a prostate cancer antigen, and will permit the comparison of different vaccine strategies (peptide-based and DNA-based) against a single antigen in human clinical trials.

**References:**

None

## Appendices:

- ❑ *Curriculum vitae*
- ❑ **McNeel DG**, Nguyen LD, Storer BE, Vessella R, Lange PH, and Disis ML. (2000) "Antibody immunity to prostate cancer-associated antigens can be detected in the serum of patients with prostate cancer." *J. Urol.* **164**:1825-1829.
- ❑ **McNeel DG**, Disis ML. (2000) "Tumor vaccines for the management of prostate cancer." *Arch. Immunol. Ther. Exp.* **48**:85-93.
- ❑ **McNeel DG**, Nguyen LD, Disis ML. (2001) "Identification of T helper epitopes derived from prostatic acid phosphatase." *Cancer Res.* **61**:5161-5167.
- ❑ **McNeel DG**, Nguyen LD, Ellis WJ, Higano CS, Lange PH, Disis ML. (2001) "Naturally occurring prostate cancer antigen-specific T cell responses of a Th1 phenotype can be detected in patients with prostate cancer." *Prostate* **47**:222-229.

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12/1992 Ph.D. Biochemistry, Molecular Biology; University of Chicago, Chicago, IL  
6/1994 M.D. University of Chicago – Pritzker School of Medicine, Chicago, IL

**Postgraduate Training:**

1994 – 1996 Residency in Internal Medicine, University of Washington, Seattle, WA  
1996 – 1997 Clinical Fellow in Oncology, University of Washington, Seattle, WA.  
1997 – 1999 Senior Fellow, Division of Medical Oncology, University of Washington, Seattle, WA. Laboratory of Dr. Mary L. (Nora) Disis

**Faculty Positions:**

1999 – 2001 Acting Instructor, Division of Oncology, University of Washington, Seattle, WA  
2001 – present Assistant Professor, Section of Medical Oncology, University of Wisconsin, Madison, WI

**Hospital Positions Held:**

1999 – 2001 Staff attending physician, University of Washington Medical Center  
2001 – present Staff attending physician, University of Wisconsin Medical Center

**Honors and Awards:**

Valedictorian, Coeur d'Alene Sr. High School, Coeur d'Alene, ID, 1982  
Honors at Entrance, Whitman College, Walla Walla, WA, 1982  
*Phi Beta Kappa*, Whitman College, 1985  
Mortar Board, Whitman College, 1986  
Outstanding Senior Chemistry Award – American Chemical Society, Whitman College, 1986  
Graduation *summa cum laude*, 1986  
Honors in major study, Chemistry, 1986 Thesis: "Purification of a ssDNA-binding protein from beef heart mitochondria"  
Honors in major study, Music, 1986 Thesis: "The origin and development of the toccata"  
Medical Scientist Training Program Award, 1987-1994  
American Cancer Society Research Grant, University of Chicago, 1990-1991  
American Association for Cancer Research-AFLAC Young Investigator Award, 1999  
Recipient, Berlex Oncology Foundation Research Fellowship Award, 1999  
Recipient, American Cancer Society Postdoctoral Fellowship Award, 1999  
Recipient, Department of Defense Postdoctoral Fellowship Award in Prostate Cancer, 1999  
American Association for Cancer Research-AFLAC Young Investigator Award, 2000  
American Association for Cancer Research-AFLAC Scholar in Training Award, 2001

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Oncology, Board Eligible, 1999

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2001                Wisconsin state

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American Association for Cancer Research, Active Member  
American Medical Association, Associate Member  
American Society of Clinical Oncology, Associate Member  
American Society of Gene Therapy, Associate Member

**Bibliography:**

**A. Peer-Reviewed Publications of Original Work:**

1. **McNeel DG**, Nguyen LD, Disis ML. (2001) "Identification of T helper epitopes derived from prostatic acid phosphatase." *Cancer Res.* **61**:5161-5167.
2. **McNeel DG**, Nguyen LD, Ellis WJ, Higano CS, Lange PH, Disis ML. (2001) "Naturally occurring prostate cancer antigen-specific T cell responses of a Th1 phenotype can be detected in patients with prostate cancer." *Prostate* **47**:222-229.
3. **McNeel DG**, Nguyen LD, Storer BE, Vessella R, Lange PH, and Disis ML. (2000) "Antibody immunity to prostate cancer-associated antigens can be detected in the serum of patients with prostate cancer." *J. Urol.* **164**:1825-1829.
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5. Disis ML, Schiffman K, Gooley TA, **McNeel DG**, Rinn K and Knutson KL. (2000) "Delayed type hypersensitivity response (DTH) is a predictor of peripheral blood T cell immunity after HER-2/neu peptide immunization." *Clin. Cancer Res.* **6**(4):1347-1350.
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7. **McNeel DG** and Tamanoi F. (1992) "Terminal region recognition factor 1, a DNA-binding protein recognizing the inverted terminal repeats of the pGKI linear DNA plasmids." *Proc. Natl. Acad. Sci. USA* **88**:11398-11402.

**B. Invited Review Articles:**

1. Disis ML, Knutson KL, **McNeel DG**, Davis D, and Schiffman K. (2001) "Clinical translation of peptide-based vaccine trials: The HER-2/neu model." *Crit. Rev. Immunol.* (in press).
2. **McNeel DG**, Disis ML. (2001) "Peptide vaccination for cancer." *Meth. Mol. Med.* (in press).
3. **McNeel DG**, Disis ML. (2000) "Tumor vaccines for the management of prostate cancer." *Arch. Immunol. Ther. Exp.* **48**:85-93.
4. Disis ML, **McNeel DG**, Rinn K, Schiffman K, and Knutson K. (1999) "Peptide-based tumor vaccines." *Curr. Opin. Oncol. Endo. Metabol. Invest. Drugs* **1**:253-259.

**C. Manuscripts Submitted:**

**D. Manuscripts in Preparation:**

1. Disis ML, **McNeel DG**. "Rat neu specific cytotoxic T cells can be generated by DNA immunization with soluble cytokine as an adjuvant."

2. **McNeel DG**, Knutson KL, Disis ML. "HLA-A2 CTL epitopes from prostatic acid phosphatase identified by ELISPOT."

**E. Submitted and Published Abstracts Presented at National Meetings:**

1. **McNeel DG**, Nguyen LD, Cardon K, Nelson PS, Gottesman, J, Disis ML (2001) "Identification of two serologic tumor antigens in patients with prostate cancer." *Proc. Amer. Assn. Cancer Res.* **42**:156.
2. **McNeel DG**, Knutson KL, Disis ML (2001) "Identification of PAP-specific MHC class I peptide epitopes by screening patients with prostate cancer by IFN $\gamma$  ELISPOT." *Proc. Amer. Assn. Cancer Res.* **42**:277.
3. **McNeel DG**, Nguyen LD, Disis ML (2000) "T cells derived from patients with prostate cancer secrete IFN $\gamma$  in response to stimulation with prostate cancer antigens." *Proc. Amer. Assn. Cancer Res.* **41**:880.
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6. **McNeel DG** and Disis ML. (1999) "T cell immunity specific for prostatic acid phosphatase (PAP) results in a destructive prostatitis in a rodent model." *Proc. Amer. Assn. Cancer Res.* **40**:256.
7. **McNeel DG** and Tamanoi F. (1991) "pGK12 from the yeast DNA 'killer' plasmid system encodes a small, basic DNA-binding protein which recognizes the inverted terminal repeats of both pGK11 and pGK12." *Eukaryotic DNA Replication Meeting*, Cold Spring Harbor, NY.

## ANTIBODY IMMUNITY TO PROSTATE CANCER ASSOCIATED ANTIGENS CAN BE DETECTED IN THE SERUM OF PATIENTS WITH PROSTATE CANCER

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### ABSTRACT

**Purpose:** Several immune based therapies targeting prostate cancer associated proteins are currently undergoing clinical investigation. In general, however, little is known about the immunogenicity of prostate cancer or which prostate cancer associated proteins elicit immune responses. We determine whether patients with prostate cancer have antibody immunity to known prostate cancer associated proteins, what the prevalence of this immunity is and whether immunity to individual proteins is associated with the stage of disease.

**Materials and Methods:** We evaluated the inherent humoral immune response against prostate specific antigen (PSA), prostatic acid phosphatase, p53 and HER-2/neu, all known prostate cancer associated proteins, in 200 patients with various stages of disease and male controls.

**Results:** Antibody immunity to PSA was significantly different between the patient (11%, 22 of 200) and control populations (1.5%, 3 of 100,  $p = 0.02$ ), and titers 1:100 or greater were particularly prevalent in the subgroup of patients with androgen independent disease (11%, 6 of 56). Antibody immunity to prostatic acid phosphatase and p53 was detected (5.5%, 11 of 200 and 6%, 12 of 200), and was not different from the control population (4%, 4 of 100,  $p = 0.57$  and 7%, 7 of 100,  $p = 0.74$ ). Antibody immunity to HER-2/neu was significantly higher in patients with prostate cancer (15.5%, 31 of 200) compared to controls (2%, 2 of 100,  $p = 0.0004$ ), and titers 1:100 or greater were most prevalent in the subgroup of patients with androgen independent disease (16%, 9 of 56).

**Conclusions:** These findings suggest that prostate cancer is an immunogenic tumor. Moreover, for PSA and HER-2/neu the prevalence of antibody immunity was higher in patients with androgen independent disease, indicating that even patients with advanced stage prostate cancer can have an immune response to their tumor.

**KEY WORDS:** antibodies, immunity, prostatic neoplasms, prostate-specific antigen

Several prostate cancer antigens are under clinical investigation as potential targets for immune based treatments of prostate cancer, including prostate specific antigen (PSA)<sup>1,2</sup> and prostatic acid phosphatase (PAP).<sup>3</sup> Despite the fact that clinical prostate cancer vaccine trials are under way, little is known about the inherent immunogenicity of prostate cancer. In fact, it has been suggested that the prostate gland is immunologically favored, given the purported immunosuppressive characteristics of the seminal fluid<sup>4</sup> and the supposed absence of a lymphatic system within the prostate.<sup>5</sup> Moreover, research performed nearly 20 years ago suggests that cell mediated immune responses are depressed in patients with prostate cancer, as judged by lymphocyte proliferation in response to mitogens, delayed type of hypersensitivity skin testing to dinitrochlorobenzene and T cell aggregation studies.<sup>6,7</sup> Later studies implicate the loss of MHC class I expression as a mechanism of immune escape by prostate cancer cells.<sup>8,9</sup>

Within the last 20 years several proteins have been identified

whose expression is essentially limited to the prostate, including PSA and PAP, and several other proteins have been identified likely involved in the metastatic progression of prostate cancer. Unlike melanoma, however, in which tumor antigens have been identified based on their inherent immunogenicity,<sup>10-12</sup> to our knowledge there have been no reports on the immunogenicity of these prostate cancer associated proteins.

We address the question of the inherent immunogenicity of prostate cancer by looking at antibody immunity to PSA, PAP, p53 and HER-2/neu, which are prostate cancer associated proteins that are known tumor antigens. PSA and PAP were chosen as the 2 best characterized prostate specific proteins. There has been a report of a probable humoral immune response to PSA occurring in patients with metastatic prostate cancer,<sup>13</sup> and others have demonstrated that it is possible to culture human cytotoxic T lymphocytes in vitro specific for PSA<sup>14,15</sup> and PAP,<sup>16</sup> suggesting that tolerance to these proteins might be circumvented in vivo. p53 and HER-2/neu were chosen as biologically relevant proteins, each implicated in the metastatic progression of prostate cancer and each previously shown to be tumor antigens in breast cancer.<sup>17,18</sup>

In our study we detected an antibody response to 1 or more of these 4 proteins in 37% (74 of 200) of patients compared with 16% (16 of 100) of male controls. Antibody responses to PAP (5.5%, 11 of 200) and p53 (6%, 12 of 200) were not

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significantly different from the control populations but antibody responses to PSA (11%, 22 of 200) and HER-2/neu (15.5%, 31 of 200) were significantly different from the control populations ( $p = 0.02$  and  $p = 0.0004$ , respectively). Higher titer responses were most prevalent in patients with androgen independent prostate cancer, although this did not reach statistical significance for PSA. These findings indicate that prostate cancer is an immunogenic tumor, and that even patients with advanced stage prostate cancer can mount an antibody immune response to proteins expressed by the tumor.

#### METHODS

**Patient populations.** With informed consent, sera were obtained from 200 patients with prostate cancer at the University of Washington Medical Center between 1997 and 1999. Sera were grouped according to disease stage and treatment status. Pretreatment sera were drawn within 1 week of surgery from 48 patients undergoing radical prostatectomy (group 1). Group 2 consisted of 55 patients with stage B or C tumors, who had been treated with radical prostatectomy or brachytherapy and had no evidence of disease recurrence. These sera were collected at a variety of times following surgery. Group 3 included 41 patients with stage D prostate cancer, on androgen ablative therapy and with evidence of androgen responsive disease by PSA monitoring. Group 4 consisted of 56 patients with metastatic, androgen independent prostate cancer. Sera in these latter 2 groups were drawn at different times in the treatment course of individual subjects. Control sera were obtained from male volunteer blood donors 37 to 73 years old, without histories of prostate or other cancers, who contributed blood products at the Puget Sound Blood Bank. All sera were stored in aliquots at  $-20^{\circ}\text{C}$  until used.

**Detection of PAP and PSA specific antibody responses in patients with prostate cancer by enzyme-linked immunosorbent assay (ELISA).** Antibodies recognizing PAP and PSA were detected by ELISA using highly purified proteins. We added  $2\text{ }\mu\text{g/ml}$  purified protein in  $50\text{ mM}$  sodium carbonate buffer (pH 9.6) to experimental wells overnight at  $4^{\circ}\text{C}$ . Plates were then blocked with phosphate buffered saline/1% bovine serum albumin for 1 hour at room temperature. After washing with phosphate buffered saline/0.1% Tween-20, plates were then probed with human sera at concentrations of 1:25, 1:50, 1:100 and 1:200 for 1 hour at room temperature, with serum added to experimental and blank wells. Serum from a patient with previously documented antibodies to PAP and PSA was included on each plate as a positive control and to serve as an interplate control. After a 1-hour incubation plates were again washed and a peroxidase-conjugated sheep anti-human Ig antibody diluted 1:5,000 in phosphate buffered saline/1% bovine serum albumin was added. Following a 45-minute incubation at room temperature, the plates were washed and developed with tetramethylbenzidine peroxidase substrate according to manufacturer instructions.

Reactions were monitored at optical density of 650 nm. and stopped with addition of hydrochloric acid to 0.5 N. concentration when the optical density of the positive control sera reached 0.3. The plates were then read at optical density of 450 nm. and the change in optical density was determined as the difference between the optical density of experimental and blank wells. Titers were calculated using a cutoff value determined from the mean + 2 standard deviations (SD) of the control population change in optical density values at the most concentrated sera dilution used (1:25). The change in optical density of the positive control sera varied by less than 1 SD among plates, and titered at 1:200 for PSA and 1:400 for PAP. All titers greater than 1:50 were confirmed in an independent assay.

**Detection of p53 specific antibody responses in patients with**

**prostate cancer by ELISA.** Antibodies recognizing nonmutated p53 were detected using a capture ELISA method, similar to that previously reported.<sup>19</sup> Briefly, 96-well ELISA plates were incubated overnight at  $4^{\circ}\text{C}$  with a mouse monoclonal antibody specific for human p53 in  $50\text{ mM}$  sodium carbonate buffer (pH 9.6). Plates were then blocked as described previously, and a lysate from a p53 abundant human breast cancer cell line, BT20, was added to experimental wells as a source of antigen. After incubation, plates were then washed, probed with human sera at concentrations of 1:25, 1:50, 1:100 and 1:200, and developed as described previously. Serum from a single patient with previously documented antibodies to p53 was included on each plate as a positive, interplate control. Titers were calculated as described previously.

**Detection of HER-2/neu-specific antibody responses in patients with prostate cancer by ELISA.** Capture ELISA was performed as previously reported,<sup>18</sup> using an IgG specific secondary antibody. As for the other ELISA assays, serum from a single patient with a previously documented IgG antibody response to HER-2/neu was included on each plate as a positive control and to serve as an interplate control.<sup>18</sup> Likewise, all titers greater than 1:50 were confirmed in an independent assay. Previous studies have shown that antibody responses with titers 1:100 or greater are not found in noncancer control sera.<sup>18</sup> Statistical comparison of study populations was performed using a chi-square test, with  $p \leq 0.05$  considered statistically significant.

#### RESULTS

**Antibody immunity to PSA can be detected in the serum of patients with prostate cancer.** Sera from 200 patients with various stages of prostate cancer and from 100 volunteer male control blood donors were screened for the presence of antibodies to PSA as described (fig. 1). Detectable titers (1:50 or greater) were found in 22 (11%) of 200 patients, which was statistically different from the control population (3%, 3 of 100,  $p = 0.02$ ). The difference between patients and controls was more striking at titers 1:100 or greater, as 6.5% (13 of 200) of patients had detectable antibodies compared with none of the controls (0 of 100,  $p = 0.009$ ). Analysis by stage of disease also suggested that antibody responses were most common in patients with androgen independent disease (6 of 56 with titers 1:100 or greater, 10.7%). However, this sub-

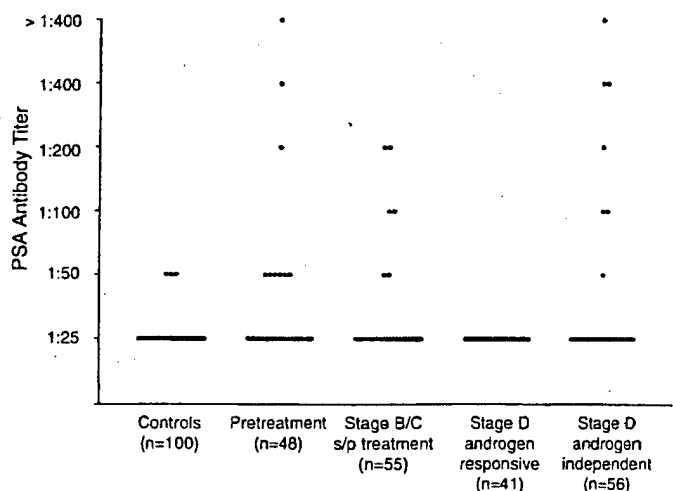


FIG. 1. Antibody immunity to PSA can be detected in serum of patients with prostate cancer. Sera from 200 patients with various stages of prostate cancer and 100 volunteer male blood donors were screened for presence of antibodies to PSA by ELISA. Individual dots represent titers determined for all individual patients or controls. s/p, status post.

group of patients was not statistically different from the combined other subgroups of patients ( $p = 0.13$ ). No correlation was noted between the presence of antibodies and serum level of PSA (data not shown). Most of the antibody responses detected were of low titer (1:50 to 1:200) but a few patients had high titer antibody responses (fig. 2). In addition, 2 patients with detectable antibodies to PSA also had detectable antibodies to either PAP or HER-2/neu.

*Patients with prostate cancer have antibodies to PAP.* Of the 200 patients 11 (5.5%) had antibody immunity to PAP (fig. 3), which was not different from the control population (4 of 100, 4%,  $p = 0.57$ ). No differences were noted with respect to stage of disease. Of interest, however, the patient with the highest antibody titer to PAP also had detectable antibodies to PSA.

*Patients with prostate cancer have antibodies to p53.* Of the 200 patients 12 (6%) and of the 100 controls 7 (7%) had low titer antibody immunity to p53 (fig. 4), which was not statistically different ( $p = 0.74$ ). No differences were noted with respect to disease stage.

*Antibody immunity to HER-2/neu can be detected in the serum of patients with prostate cancer and is most prevalent in patients with androgen independent disease.* Sera from the 200 patients with prostate cancer were screened for antibodies to HER-2/neu using the titer based assay described previously,<sup>18</sup> and the results were compared with 100 controls. Overall, 31 (15.5%) of 200 patients had detectable antibodies to HER-2/neu compared with 2 (2%) of 100 controls ( $p = 0.0004$ ) at titers 1:50 or greater (fig. 5). At titers 1:100 or greater, at which it has previously been reported that antibody responses are not detected in patients without cancer,<sup>18</sup> 8.5% (17 of 200) of the patient population had detectable antibodies ( $p = 0.003$ ). At this titer HER-2/neu-specific antibody responses were most prevalent in patients with androgen independent disease (9 of 56, 16%) compared with the other combined subgroups of patients (8 of 144, 5.6%,  $p = 0.02$ ). As indicated previously, 1 patient with advanced stage disease and antibodies to HER-2/neu also had detectable antibodies to PSA.

#### DISCUSSION

There is currently much enthusiasm about the use of immune based therapies for the treatment of prostate can-

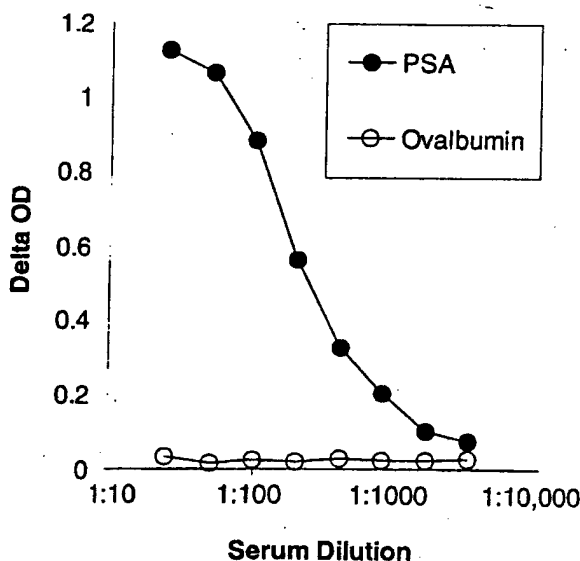


FIG. 2. Some patients have high titer antibody responses to PSA. Results of ELISA detecting antibodies to either PSA or ovalbumin (negative control) in patient with metastatic androgen independent prostate cancer. PSA antibodies titrated at 1:1,600. OD, optical density.

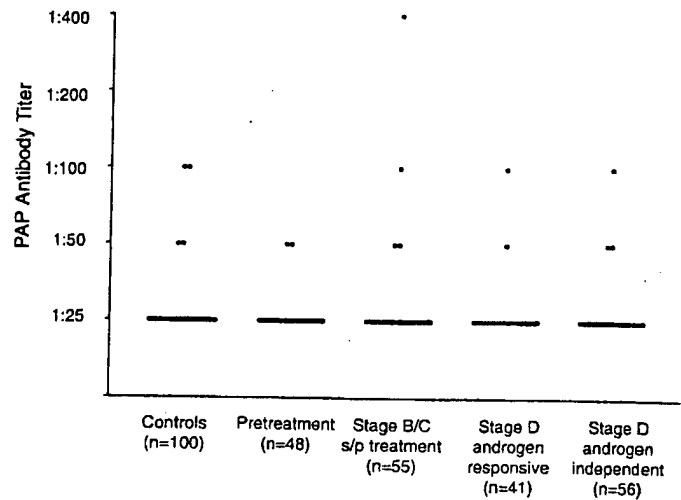


FIG. 3. Rare patients with prostate cancer have antibodies to PAP. Sera from 200 patients with various stages of prostate cancer and 100 volunteer male blood donors were screened for presence of antibodies to PAP. Dots represent titers determined for all individual patients or controls. s/p, status post.

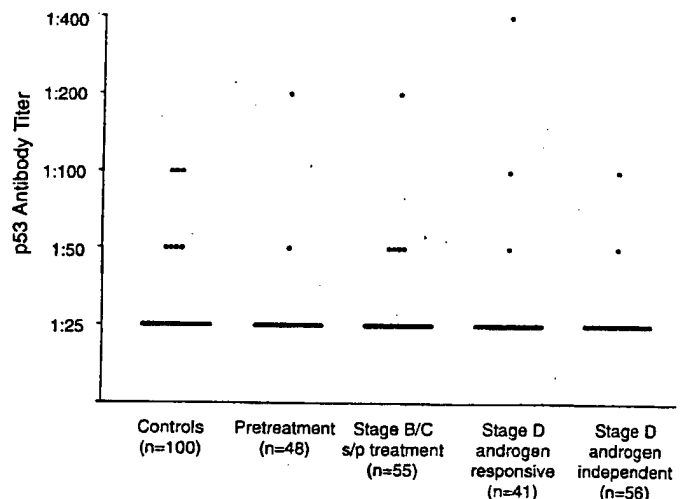


FIG. 4. Patients with prostate cancer have antibodies to p53. Sera from 200 patients with various stages of prostate cancer and 100 volunteer male blood donors were screened for presence of antibodies to p53. Dots represent titers determined for individual patients or controls. s/p, status post.

cer. Unlike melanoma, however, in which tumor associated antigens have been defined based on the endogenous immune response to particular proteins,<sup>11,20</sup> there has been little investigation of the immunogenicity of prostate cancer associated proteins. We report an overview of the humoral immunogenicity of 4 prostate cancer associated proteins, 2 of which are essentially prostate specific, and 2 of which are biologically relevant to the progression of prostate cancer and have been shown to elicit immune responses in patients with other tumors. The specific questions we addressed were 1) do patients with prostate cancer have antibody immunity to known prostate cancer associated antigens, 2) what is the prevalence of this immunity and 3) is immunity to individual proteins associated with the stage of disease.

Patients with prostate cancer can have antibody immunity to prostate cancer associated proteins. While there were no cancer specific humoral responses detected to PAP or p53, antibody responses to PSA and HER-2/neu were significantly different from a control population. It is unclear why there would be more antibody responses detected to PSA than PAP, given that they are both prostate specific proteins. This find-

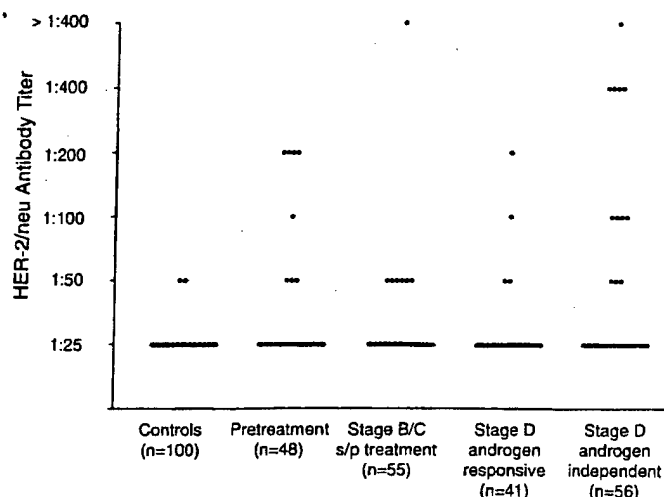


FIG. 5. Antibody immunity to HER-2/neu can be detected in serum of patients with prostate cancer, and is most prevalent in patients with androgen independent disease. Sera from 200 patients with various stages of prostate cancer and 100 male controls were screened for presence of antibodies to HER-2/neu by capture ELISA. Dots represent titers determined for all patients and control blood donors. s/p, status post.

ing may be a reflection of expression levels of these proteins, with typically higher serum levels of PSA than PAP detected in patients with metastatic disease, and consequently perhaps greater uptake and antigen presentation. On the other hand, this finding may reflect the inherent humoral immunogenicity of these specific proteins, with perhaps higher tolerance to PAP, which may share epitopes with other tissue phosphatases than PSA. In any case the antibody responses detected were generally low titer, although a few patients had high titer antibodies to PSA or HER-2/neu. The presence of antigen specific immunity detected only in patients with cancer suggests that it is the presence of tumor itself that elicits an immune response. Moreover, the fact that the responses to HER-2/neu were IgG indicates the likely presence of antigen specific CD4<sup>+</sup> T cell responses mediating immunoglobulin class switch and participating in this immune response.

There was a high prevalence of humoral immune responses to the proteins tested in patients with prostate cancer at titers 1:50 or greater. Of the 200 patients 26% had detectable antibodies to PSA and/or HER-2/neu compared to only 5% of the control population. The prevalence of HER-2/neu-specific antibodies with titers 1:100 or greater, particularly in patients with androgen independent prostate cancer (16%), was similar to what has previously been reported in other adenocarcinomas, such as early stage breast cancer (11%)<sup>18</sup> and colorectal carcinoma (14%).<sup>21</sup> In contrast, autoantibodies to p53 have been detected in 24% to 30% of patients with ovarian, breast and lung cancers.<sup>17,19,22</sup> Our results are consistent with studies by Lang et al, who reported a low incidence (3%) of p53 autoantibodies in prostate adenocarcinomas compared with other urogenital tumors.<sup>23</sup> This low prevalence of p53 autoantibodies may be a result of less homogeneous, nuclear accumulation of p53 in prostate cancer compared with other adenocarcinomas.<sup>24</sup>

In general, we found that the majority of humoral immune responses occurred in patients with androgen independent prostate cancer. The observation that the majority of responses were in patients with late stage disease may reflect that these low level immune responses occur as a result of shed antigens in more bulky disease. Despite the mechanism, however, this observation suggests that even patients with metastatic disease are able to mount an immune response to the tumor.

Tumor antigen specific cytotoxic T lymphocytes have long been considered critical final effectors in an effective antitumor immune response.<sup>25</sup> Others and we have found in a rodent model that a PAP specific Th1 phenotype with PAP specific cytotoxic T lymphocytes is critical for actual destruction of prostate tissue.<sup>26,27</sup> PSA specific cytotoxic T lymphocyte responses have previously been detected in a patient with prostate cancer, suggesting that a potentially therapeutic immune response can exist in vivo.<sup>28</sup> Th1-like immune responses with IgG2 subtype antibody responses are often associated with a concurrent cytotoxic T lymphocyte response. Studies are currently under way to determine the isotype of the IgG prostate antigen specific antibody responses.

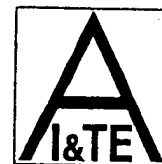
## CONCLUSIONS

Our findings suggest that prostate cancer is an immunogenic tumor, that is patients with prostate cancer can have antibody immunity to 1 or more proteins associated with the cancer. For PSA and HER-2/neu the prevalence of antibody immunity was higher in patients with end stage disease, suggesting that those with metastatic prostate cancer can have an immune response to the disease. Presently it is unknown whether there are other prostate cancer associated antigens that may be more widely immunogenic but our findings suggest that techniques that have been used in the study of melanoma and renal cell carcinoma to identify antigens recognized by the host immune system may be useful in prostate cancer to define more rational targets for immune based therapies.

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*Review*

## Tumor Vaccines for the Management of Prostate Cancer

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**Abstract.** Prostate cancer is a significant health problem and one of the leading causes of cancer-related death among men. Given the typically long natural history of the disease, there is considerable interest in developing new therapies to treat or prevent metastatic disease, and cancer vaccines are a particularly attractive immune-based approach. Early clinical studies using non-specific immunomodulatory treatments have met with limited success, but also suggest that improved immunologic approaches might be useful in treating human prostate cancer. Over the last decade, the identification of immune cells responsible for actual destruction of prostate tissue and advances in immunologic and molecular techniques have led to a variety of vaccination approaches that are currently being evaluated in human clinical trials. The present article discusses the rationale in animal models for particular immunization strategies and describes the vaccines currently being used in patients with prostate cancer. The ongoing identification of tumor antigens and proteins involved in prostate cancer progression and the development of better immunologic animal models suggest a hopeful future for the design of effective prostate cancer vaccines.

**Key words:** prostate cancer; tumor vaccines; clinical trials; immunotherapy.

Prostate cancer is a significant health problem worldwide and currently second only to skin cancer as the most diagnosed male malignancy in the United States<sup>3</sup>. After appropriate local therapy, approximately 30% of patients will develop metastatic disease and approximately 10% of patients will die of their disease. At present there is no cure for metastatic prostate cancer and no accepted adjuvant therapy proven to reduce tumor progression. There is considerable interest in developing new therapies to treat or prevent metastatic disease. Recently, gene therapy and various immune-based strategies have been explored as possible treatments for prostate cancer<sup>21, 23, 24</sup>. Given the gener-

ally long natural history of prostate cancer, tumor vaccines are a particularly attractive immune-based approach to consider for adjuvant therapy.

### Background

Initial investigations performed nearly 20 years ago suggested prostate cancer is not an immunogenic tumor. Early reports suggested the prostate gland is devoid of afferent lymphatics<sup>52</sup>. In addition, non-specific tests of immune function, such as T cell rosette formation, T cell blastogenic response to mitogen

Abbreviations used: TIL – tumor-infiltrating lymphocytes, DTH – delayed type hypersensitivity, PSA – prostate specific antigen, GM-CSF – granulocyte-macrophage colony stimulating factor, PAP – prostatic acid phosphatase, PSMA – prostate specific membrane antigen, CTL – cytotoxic T lymphocytes, MHC – major histocompatibility complex, KLH – keyhole limpet hemocyanin.

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stimulation, and delayed type hypersensitivity (DTH) responses to common recall antigens, were all found to be reduced in patients with prostate cancer<sup>19, 56</sup>. Other studies documented the reduction of major histocompatibility complex (MHC) class I molecules in metastatic prostate cancer lesions and disruption of the prostatic acid phosphatase TAP transporter machinery in human prostate cancer cell lines, suggesting possible mechanisms of escape from immune detection<sup>46</sup>. Finally, the absence of prostate tumors occurring in T cell deficient mice suggested that the presence of prostate cancer was not due to a defect in immune surveillance<sup>43</sup>. Such findings led investigators to conclude that prostate cancer is not an immunogenic tumor<sup>60</sup>.

More recent studies have challenged these assumptions. First, several reports have confirmed the presence of lymphocytic infiltrates in the prostate, suggesting the prostate is not immunologically privileged<sup>34</sup>. Second, reported cases of granulomatous prostatitis demonstrate that inflammatory reactions occur within the prostate gland<sup>28</sup>. Third, prostatic apoptosis associated with androgen ablative therapy has been shown to be, in part, mediated by an immune response in a rat model<sup>30</sup>. Fourth, the presence of tumor-infiltrating lymphocytes (TIL) in prostate cancer specimens has been associated with higher 10-year survival than the absence of TIL, suggesting cancer-specific immune responses may play a role in tumor surveillance<sup>59</sup>. Moreover, our group has demonstrated that patients with prostate cancer develop humoral immunity to prostate specific antigen (PSA) and HER-2/neu, and the prevalence of these immune responses is more common in patients with metastatic disease (McNEEL, submitted). Finally, we have also shown that patients with prostate cancer have preexistent T cell responses to PSA and PAP of a Th1-like phenotype, suggesting that potentially therapeutic immune responses already exist *in vivo*, albeit at low levels (McNEEL, submitted).

Recent advances in molecular biology and basic immunology, including the identification of cytokines responsible for promoting specific types of immune responses, an identification of the immune cell subsets responsible for eradicating tumor cells, and the development of appropriate animal tumor models, have led to a variety of new approaches to generate tumor-specific immune responses by means of tumor vaccines. The present article will discuss the rationale in animal models for particular immunization strategies and describe the immunization strategies currently being used in patients with prostate cancer. These animal models and the human vaccine trials discussed are summarized in Table 1.

## Whole Cell Vaccines

The goal of whole cell vaccines is to generate an anti-tumor immune response to a wide variety of tumor-associated antigens. Traditionally, the major difficulty with this approach has been that whole cell vaccines are typically only weakly immunogenic. Early studies in human vaccines with irradiated prostate cancer cells as vaccines showed modest evidence of an immunologic response when administered with foreign proteins<sup>5, 42</sup>. These findings led researchers to attempt to increase the immunogenicity of cellular vaccines themselves. The Dunning rat model provided a good model for study, as the MatLyLu derived cell line forms anaplastic, androgen-independent tumors that spontaneously metastasizes to the lymph nodes and lung when injected orthotopically in rats<sup>25</sup>. Studies showed that this tumor was poorly immunogenic; splenocytes from tumor-bearing animals demonstrated no cytolytic activity *in vitro* to MatLyLu cells and immunization of animals with irradiated MatLyLu cells generated no protection from subsequent exposure to live tumor cells<sup>60</sup>. Studies conducted attempted to increase the immunogenicity of such cellular vaccines by transfecting them with immunomodulatory cytokines, similar to previous studies in animal models of melanoma<sup>11, 16</sup>. Dunning rat prostate cancer cell lines, when transfected to express IL-2 and to a lesser extent granulocyte-macrophage colony stimulating factor (GM-CSF), and then used to immunize rats, protected immunized animals from subsequent challenge with live tumor cells<sup>60</sup>. Splenocytes from immunized animals showed cytolytic activity toward non-transfected MatLyLu cells<sup>60</sup>. Similar results were found by SANDA et al.<sup>45</sup> with GM-CSF transfected tumor vaccines in the Dunning rat model. This group went on to demonstrate that primary human prostate cancer cells could be transfected to express human GM-CSF<sup>45</sup>. These findings were ultimately translated to human clinical trials in renal cell carcinoma<sup>48</sup>, melanoma<sup>53</sup>, and the first report of a GM-CSF-modified autologous prostate cancer cell vaccine trial has recently been reported by SIMONS et al.<sup>49</sup>. In this study, 8 patients with large primary tumors undergoing radical prostatectomy had primary tumor lines established from specimens obtained at surgery. These lines were then transfected to express human GM-CSF, irradiated, and used to immunize patients 3 times over 3-week intervals. Eight of 11 patients were able to complete the vaccination series, 6 of whom had evidence of progressive disease, and 2 had transient clinical responses as determined by PSA serum levels. Three of the patients developed antibody responses to proteins expressed by

**Table 1.** Summary of preclinical animal models and recent human prostate cancer vaccine trials

	Animal models	Investigators	Human clinical trials	Investigators
<b>Whole cell vaccines</b>				
GM-CSF transfected tumors	Dunning rat	SANDA et al. <sup>45</sup> VIEWEG et al. <sup>60</sup>	phase I (autologous cell lines) phase II (LNCaP)	SIMONS et al. <sup>49</sup> SIMONS Cell GeneSys Inc. SLOVIN
IFN- $\gamma$ and IL-2 transfected LNCaP cell line	Dunning rat	VIEWEG et al. <sup>60</sup>	phase I/II	
<b>Dendritic cell vaccines</b>				
PSMA peptides			phase II	MURPHY et al. <sup>39</sup>
PAP recombinant protein			phase I	FONG et al. <sup>14</sup>
<b>Antigen-specific vaccines</b>				
PAP	Dunning rat vaccinia-PAP	FONG et al. <sup>15</sup>	phase I DC / protein	FONG et al. <sup>14</sup>
PSA	Primate vaccinia-PSA	HODGE et al. <sup>22</sup>	phase I/II OncoVax-P	SPITLER <sup>21</sup>
			phase I Vaccinia-PSA	SANDA <sup>47</sup>
			phase II Vaccinia/fowlpox	Kaufman ECOG
	Murine plasmid DNA	KIM et al. <sup>29</sup>		
PSMA			phase II DC / peptide	MURPHY <sup>39</sup>
Carbohydrates	Murine MUC1 - KLH	ZHANG et al. <sup>62</sup>	phase I globo H - KLH	SLOVIN <sup>51</sup>
			phase I Thompson- Friedenreich - KLH	SLOVIN <sup>50</sup>
HER-2/neu	Fischer rat MHC class II peptides neu transgenic mouse	DISIS et al. <sup>10</sup> DISIS	phase I MHC class I peptide	MCNEEL

human prostate cancer cell lines<sup>49</sup>. Of note, 2 of 8 patients had delayed type hypersensitivity (DTH) responses to autologous tumor cells prior to vaccine treatment, suggesting that patients develop either low-level or non-therapeutic immune responses to their tumors *in vivo*.

The whole cell vaccine approach has potential advantages as well as disadvantages. Given that appropriate tumor antigens have not really been identified for prostate cancer as they have for other human solid tumors, one potential advantage is that individual antigens are not necessarily targeted. The goal of cellular vaccine approaches is to generate an immune response against the most immunogenic proteins. On the other hand, this also represents a disadvantage, as immunizing with whole cells exposes the immune system to hundreds and perhaps thousands of irrelevant proteins, possibly swamping out a potentially therapeutic immune response. In addition, the transfected cytokine

itself may be immunogenic. Our group has demonstrated that immune responses to recombinant human GM-CSF can develop after vaccination<sup>36</sup>. Moreover, whole cell vaccines are laborious, costly, and of necessity tailor-made for individual patients. Such vaccine strategies may not be feasible for large-scale studies. Likewise, similar to vaccines used for the prevention of infectious diseases, the role of prostate cancer vaccines, and tumor vaccines in general, will likely be in the adjuvant setting with minimal detectable disease<sup>55</sup>. In the study reported by SIMONS et al.<sup>49</sup>, the authors note that the production of transfected tumor vaccines necessitated large primary tumors, and therefore vaccinations were restricted to patients with large, high-risk tumors. For this reason, their approach in phase II studies, and the approach of others using similar cytokine-transfected prostate cancer cellular vaccines, is to use cytokine-transfected allogeneic prostate cancer cell lines<sup>49</sup>.

## Dendritic Cell Vaccines

Dendritic cells are potent antigen presenting cells<sup>54</sup>. Investigations in murine models have shown that dendritic cells pulsed *ex vivo* with lysates from tumor cells can be used to vaccinate naive syngeneic mice, protecting animals from tumor challenge and reducing the number of metastases<sup>6, 12</sup>. These studies have demonstrated that a tumor-specific immune response can be generated by dendritic cell immunization, and have led to the initiation of human clinical trials in prostate cancer. G. Murphy and colleagues initially reported that autologous dendritic cells from patients with prostate cancer could be pulsed *ex vivo* with either autologous tumor cell lysates or an HLA-A2-restricted peptide epitope from prostate specific membrane antigen (PSMA) to generate tumor-specific or peptide-specific cytotoxic T lymphocytes (CTL) *in vitro*<sup>58</sup>. This group then went on to pioneer the use of dendritic cell vaccines for prostate cancer in an antigen-specific fashion using two putative HLA-A2-restricted MHC class I epitopes from the PSMA protein. To date, these investigators have observed some potential clinical responses in select patients, and perhaps some evidence of PSMA-specific cellular immune responses elicited by vaccinations<sup>39, 44</sup>.

In principle, using the antigen presenting cells that most directly promote cellular immune responses is a very reasonable approach. As an example, DHODAPKAR et al.<sup>7</sup> demonstrated that healthy subjects could be effectively immunized, as assessed clinically by DTH testing and by quantitative *in vitro* assays of antigen-specific T cells, with autologous dendritic cells pulsed with 1 of 3 well-characterized immunogenic proteins or peptide epitopes in a single immunization. Animal studies using dendritic cells pulsed with tumor cell extracts have demonstrated that knowledge of the specific antigens recognized need not be known, as animals immunized in this fashion develop an anti-tumor immune response<sup>12</sup>. Like whole cell vaccines, however, the generation of dendritic cells is a labor-intensive, individualized therapy that may be difficult to extrapolate to larger pools of patients. Nonetheless, this strategy is entering phase II/III studies for the treatment of advanced melanoma, and shows promise for future development of prostate cancer vaccines.

## Antigen-Specific Vaccines

Given the disadvantages and individualized therapy inherent in cellular vaccines, several investigators have focused on antigen-specific vaccines. The theoretical

advantages of targeting a specific antigen include the ability to concentrate the immune response against one specific, immunogenic target and thereby avoid exposure to extraneous or potentially disadvantageous antigens. Certainly in the case of vaccines for infectious diseases, an immune response directed against single, immunogenic proteins may be advantageous<sup>31, 40</sup>. In addition, this approach does not generally rely on culturing cells from individual patients, and therefore may be more amenable to treating larger numbers of patients in a reproducible fashion. Finally, the identification of proteins specifically involved in the metastatic progression of prostate cancer may permit vaccines to interfere directly with the progression of the disease. The identification of several prostate-specific proteins over the last several years and the ongoing elucidation of the pathways involved in prostate cancer progression, metastasis, and androgen independence make antigen-specific vaccines an attractive strategy for the future development of prostate cancer vaccines.

Several delivery systems for antigen-specific vaccines are being investigated, including recombinant bacterial and viral delivery systems, recombinant proteins in adjuvant, MHC-binding peptide epitopes, the use of various cytokines as immunomodulatory adjuvants, and bacterial plasmid DNA encoding the target antigen. Each of these approaches will be discussed in the context of the specific antigens currently being targeted in human prostate clinical trials.

## Prostatic Acid Phosphatase

The identification over the last 10 years of several proteins whose expression is essentially limited to the prostate has provided several candidate antigens for vaccine trials. PAP was first identified in 1938, and was initially used as a serum marker for the detection of prostate cancer<sup>17, 20</sup>. Given the early identification of a rat homolog, our group and others have used a rodent model to study vaccination strategies targeting this prostate-specific protein. FONG et al.<sup>15</sup> reported that immunizing rats with recombinant vaccinia virus engineered to express rat PAP was not effective in generating an immune response to PAP. By immunizing rats with vaccinia virus expressing the human homologue, however, rats generated a cross-reactive immune response with the generation of PAP-specific CTL and destruction of prostate tissue. In similar studies, we have found that immunizing rats with vaccinia-PAP constructs repeatedly does not generate a PAP-specific response, but immunizing first with vaccinia-PAP and then boost-



ing with PAP protein in adjuvant leads to a Th1 biased cellular response with the generation of destructive prostatitis<sup>35</sup>. The results of these studies have provided important information for the design of human prostate cancer vaccine strategies. First, an antigen-specific immune response is capable of destroying prostate tissue *in vivo*. Second, the effector cells are predominantly Th1-like with antigen-specific CTL; antigen-specific antibody responses did not result in destructive prostatitis. Third, repeated immunizations with a viral vector do not necessarily lead to an antigen-specific immune response in the case where a "self" protein is targeted most likely because of the overwhelming majority of foreign, more immunogenic proteins introduced by means of viral vaccination. FONG et al.<sup>14</sup> have used this information to initiate a clinical trial targeting PAP by using autologous dendritic cells pulsed *ex vivo* with the recently identified mouse PAP homolog. By this strategy, they preliminarily report that patients develop T cell responses to PAP.

### Prostate Specific Antigen

The essentially prostate-specific expression of PSA has made it a natural target for antigen-specific vaccine strategies. We have demonstrated that patients with prostate cancer, and particularly the subgroup of patients with metastatic prostate cancer, develop both a humoral and cellular immune response to PSA<sup>37</sup> (Mc NEEL, submitted). In a series of phase I/II clinical trials, Jenner Biotherapies treated 45 patients with prostate cancer with recombinant human PSA in a lipophilic adjuvant, OncoVax-P. Patients were immunized by a variety of routes (reviewed in<sup>21</sup>), including i.m., i.v., s.c. and i.d. Vaccines were also given in an oil emulsion or with a variety of immunomodulatory agents (BCG, GM-CSF, IL-2 and cyclophosphamide). To date, preliminary results suggest that patients treated develop antibodies to PSA and DTH responses to PSA<sup>21, 38</sup>.

The success of viral vectors for generating cellular immune responses has led other investigators to use vaccinia or fowlpox as a means of immunizing patients against PSA. HODGE et al.<sup>22</sup> reported the construction of a recombinant vaccinia virus expressing human PSA and its safety and efficacy in non-human primates in generating a PSA-specific antibody response. These results have led to a phase I/II trial evaluating the safety and efficacy of a recombinant vaccinia-PSA (PROST-VAC) vaccine construct in patients with stage D0 prostate cancer. SANDA et al.<sup>47</sup> have recently reported the results of this trial, in which 1 of 6 patients developed

an IgG antibody response to PSA, and one patient had stable serum PSA levels following immunization. The investigators do not report whether patients generate T cell immune responses. Given the limited number of immunizations that can be performed with vaccinia virus due to the overwhelming immune response to the vector itself, investigators have initiated a phase II cooperative study using either recombinant fowlpox-PSA or fowlpox-PSA with vaccinia-PSA in a prime-boost strategy.

There are multiple reports in animal models that immunization strategies using bacterial plasmid DNA alone as the delivery system is a potent means of generating cellular immune responses, particularly CTL responses<sup>57</sup>. Advantages of DNA immunization are that it essentially removes exposure to other competing antigens expressed in viral immunization strategies and is not MHC-restricted as are peptide vaccination strategies. In an animal model, KIM et al.<sup>29</sup> have used plasmid DNA encoding human PSA under a eukaryotic expression promoter as a means of immunizing mice. This strategy was effective at generating both humoral and CTL responses specific for PSA. Human prostate cancer vaccine trials using DNA as the antigen delivery system are being contemplated.

One of the limitations to vaccine strategies targeting PSA is that serum PSA levels are increasingly becoming accepted as a surrogate clinical endpoint in prostate cancer treatment trials<sup>2</sup>. Monitoring PSA levels may not be a suitable clinical endpoint in vaccine trials in which immune responses are generated to this protein. Another limitation has been the absence of relevant animal models, since mice and rats do not have a PSA homologue. WEI et al.<sup>61</sup> have reported the development of a transgenic mouse model that expresses human PSA in a prostate-restricted fashion. This model, and similar transgenic animal models, will provide valuable systems to evaluate the safety and potential efficacy of different vaccine strategies targeting PSA and other antigens in a preclinical fashion.

### Prostate Specific Membrane Antigen

PSMA was originally identified and cloned as the protein recognized by a monoclonal antibody raised against the human metastatic prostate cancer cell line LNCaP<sup>27</sup>. Ribonuclease protection studies demonstrated that expression of the protein is nearly restricted to the prostate, and malignant prostate tissue in general highly expresses the protein<sup>26</sup>. The presence of a membrane-bound, prostate-specific protein suggested it

might be a good protein for diagnostic imaging studies to detect prostate cancer, as well as a target antigen for immunotherapeutic strategies. As described above, G. Murphy and colleagues reported that dendritic cells from prostate cancer patients could be pulsed *ex vivo* with an HLA-A2-restricted peptide epitope from PSMA to generate peptide-specific CTL<sup>58</sup> and are conducting several clinical trials using dendritic cells to generate immunity to PSMA.

### Cancer-Associated Membrane Carbohydrates

Specific membrane-bound carbohydrate moieties, such as GM2, MUC1, globo H and Thompson-Friedenreich antigen, have been found to be expressed preferentially on the surface of a variety of different tumor cells, suggesting that they may be candidate targets for immunotherapeutic approaches<sup>32</sup>. Investigators at Memorial Sloan-Kettering Cancer Center screened primary and metastatic human prostate tissues compared with a panel of normal tissues by immunohistochemistry and identified several membrane-bound carbohydrate antigens that are overexpressed on malignant prostate tissue compared with normal tissues<sup>63</sup>. They went on to initiate a phase I vaccine trial in prostate cancer targeting the globo H hexasaccharide, a membrane-bound carbohydrate molecule<sup>51</sup>. In this study, patients were immunized subcutaneously monthly for 5 months with globo H hexasaccharide conjugated to the keyhole limpet hemocyanin (KLH) carrier antigen in an immunologic adjuvant, QS-21. A similar immunization strategy had been used previously in patients with melanoma targeting the GM2 ganglioside antigen, with evidence of IgG antibody responses being generated to GM2 with this approach<sup>33</sup>. In the globo H study, the investigators report IgM antibody responses to globo H and stable serum PSA slopes compared with pre-treatment PSA slopes in specific patients over a 2-year period, suggesting that such treatment may have a clinical effect. A new study targeting another carbohydrate antigen, the Thompson-Friedenreich antigen, conjugated to KLH in QS-21 adjuvant, is underway, but no results are yet available<sup>50</sup>.

### HER-2/neu

HER-2/neu is well documented as a tumor-associated antigen in human breast, ovarian, and colon cancer<sup>1, 8, 13</sup>. Our group has previously reported that patients with early stage breast cancer have preexistent

antibody and T cell responses to HER-2/neu that are not detected in a control population<sup>8</sup>. These responses are low-level, but suggest that immune responses to HER-2/neu can be generated *in vivo*. Relevant animal models demonstrated that immunization of rats with either the human homologue or with MHC class II-binding peptides derived from rat PAP are capable of eliciting rat *neu*-specific T cell responses<sup>10</sup>. In a *neu* transgenic mouse model of human breast cancer, immunization of mice with MHC class II-binding peptides with GM-CSF as an adjuvant is not only capable of eliciting anti-*neu* protein-specific T cell responses, but in protecting immunized animals from developing tumors (unpublished data). These results have led to a human clinical trial targeting HER-2/neu using MHC class II-binding peptides with GM-CSF as an adjuvant. In an interim summary, 8 of 8 patients developed peptide-specific T cell immunity to the immunizing peptides and 6 of 8 developed HER-2/neu-specific T cell immunity<sup>9</sup>.

The role of HER-2/neu in prostate cancer progression has been unclear, with different groups reporting differing findings about the expression of HER-2/neu in primary prostate cancers<sup>18, 41</sup>. Recent data, however, suggests that HER-2/neu overexpression may permit androgen-independent growth of prostate cancer cells<sup>4</sup>. We have reported that patients with prostate cancer, and in particular the subgroup of patients with androgen-independent prostate cancer, have preexisting antibody immunity to HER-2/neu, similar to HER-2/neu-overexpressing breast cancer (McNEEL, submitted). Given these findings, and based on our animal model data that a CTL response is critical for mediating destruction of prostate tissue, we have initiated a phase I vaccine trial using a well-characterized HLA-A2-restricted 9-mer MHC class I-binding epitope (p369) derived from the amino acid sequence of HER-2/neu<sup>1, 13</sup>. In this trial, peptide is administered intradermally with either GM-CSF as a vaccine adjuvant, or in the course of flt3 ligand stimulated dendritic cell mobilization. Flt3 ligand is a potent growth and differentiation factor for dendritic cells, and consequently the purpose of the study is to evaluate whether dendritic cells expanded *in vivo* are capable of properly presenting peptide antigen to generate an antigen-specific CTL response.

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# Identification of T Helper Epitopes from Prostatic Acid Phosphatase<sup>1</sup>

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## ABSTRACT

Helper T cells (Th cells) play a central role in the initiation and maintenance of immune responses, including antitumor immunity. The ability of Th cells in murine models to maintain and enhance the cytolytic efficacy of CD8<sup>+</sup> CTLs has led to a renewed interest in identifying human tumor antigens recognized by Th cells. Prostatic acid phosphatase (PAP) is a prostate cancer-associated tumor antigen. A rodent model has demonstrated that PAP-specific CTLs can induce destructive prostatitis. Human MHC class I epitopes derived from PAP have been identified previously, and peptide-specific CTLs have been shown to be able to lyse an MHC-restricted prostate cancer cell line. In the current study, we sought to identify Th epitopes derived from PAP that might be used to elicit PAP-specific Th responses, ultimately in the context of human vaccines targeting PAP. Using peripheral blood mononuclear cells (PBMCs) from subjects with and without PAP-specific Th responses, we screened a panel of 10 potential peptide epitopes for peptide-specific T-cell proliferation. Four peptides, p81-95, p199-213, p228-242, and p308-322, were identified for which peptide-specific T-cell proliferation occurred in the majority of patient PBMC samples that also exhibited PAP-specific T-cell proliferation. PBMCs from patients with prostate cancer and without PAP-specific Th immunity were then cultured *in vitro* with these four peptides. Peptide-specific T-cell lines could be generated from two of the four peptides, p199-213 and p228-242, that also proliferated in response to PAP protein stimulation. The ability of these two peptides to elicit PAP-specific Th responses suggests that they represent naturally processed PAP-specific MHC class II epitopes.

## INTRODUCTION

Th cells,<sup>3</sup> typically CD4<sup>+</sup> T cells, play a central role in the initiation of immune responses. Depending on the nature of the Th cells elicited, an immune response may be predominantly tolerizing or inflammatory (1). This central role is of particular significance in antitumor immune responses, in which antigens recognized are typically autologous proteins, and therefore, the balance of inflammatory and tolerant immunity is of great importance (2, 3). Animal models have demonstrated the importance of CD4<sup>+</sup> T cells in recruiting CD8<sup>+</sup> CTLs (4) and other inflammatory cells such as macrophages and eosinophils (5). CTLs have generally been believed to be the major participants in actual destruction of malignant tissue, and animal models suggest that the absence of a concurrent antigen-specific Th response can lead to CTL tolerance or ineffectiveness (1, 6). Other murine studies (7) have demonstrated that CD4<sup>+</sup> T cells are required to maintain CTL memory and survival, as well as their functional ability to infiltrate tumors (8). Similar results in human *in vitro* studies (9) have demonstrated that CD4<sup>+</sup> T cells cross-prime dendritic cells to elicit tumor-specific CTLs. These roles of Th cells in antitumor

immunity have led to a recent interest to define human tumor antigens recognized by Th cells (3, 10, 11). To date, CD4<sup>+</sup> T-cell responses have been identified specifically for several melanoma tumor antigens, including tyrosinase (12), gp100 (13), and MAGE-3 (14). For prostate cancer, few human tumor antigens recognized by Th cells have been identified, and even less is known about the peptide epitopes recognized by these cells (15, 16).

Studies (17-19) in a rodent model of prostatitis have demonstrated that a CTL response directed at the prostate can destroy prostate tissue *in vivo*. These findings have led to an increased enthusiasm for the development of vaccines for treating patients with prostate cancer, specifically designed to elicit CTL immune responses to destroy malignant prostate cancer cells (20-22). One means of eliciting CD8<sup>+</sup> CTLs has been to immunize directly with antigen-specific MHC class I peptide epitopes recognized by CD8<sup>+</sup> T cells. To that end, several groups have identified MHC class I epitopes from different potential prostate tumor antigens, including prostate-specific antigen (16, 23-25), prostate-specific membrane antigen (16, 26), and PAP (27), with the concept of using these peptides as vaccine antigens. Because of the critical role of CD4<sup>+</sup> T cells in eliciting effective CTLs and maintaining a CTL memory response, others (11, 28-30) have advocated the incorporation of Th epitopes into the design of CTL epitope immunization strategies. Murine studies (31, 32), in fact, suggest that immunization with MHC class I peptide epitopes requires additional CD4<sup>+</sup> T-cell help to elicit effective CTL responses. Consequently, identification of Th epitopes to defined tumor antigens may be important for inclusion in MHC class I peptide-based vaccines for prostate cancer, as well as other tumors, to enhance an antigen-specific CTL responses (11, 13, 16, 33, 34).

PAP is a potential tumor-associated antigen in prostate cancer, given that expression of PAP is essentially restricted to prostate tissue (35, 36), including metastatic prostate cancer (37, 38). Studies (19) in rats have demonstrated that a CTL response directed at the rat PAP homologue will destroy prostate tissue *in vivo*. As noted above, investigators have identified, in a human *in vitro* system, several HLA-A2 peptide epitopes derived from human PAP. PAP peptide-specific CTLs can be cultured from the peripheral blood of volunteer male blood donors and are able to lyse an MHC-restricted prostate cancer cell line (27). The use of CTL peptide epitopes in a human vaccine study to elicit PAP-specific CTLs has been contemplated (27). In the current study, we sought to identify PAP-specific Th peptide epitopes that might be used to elicit a PAP-specific Th response in the context of a peptide-based vaccine. We have identified previously (15) subjects with prostate cancer who have PAP-specific Th responses. Using PBMCs from subjects with and without PAP-specific Th responses, we screened a panel of 10 potential peptide epitopes for peptide-specific T-cell proliferation. Four peptides were identified for which peptide-specific T-cell proliferation occurred in the majority of patient PBMC samples that also exhibited PAP-specific T-cell proliferation. PBMCs from patients with prostate cancer and without PAP-specific T-cell responses were then cultured *in vitro* with each of these four peptides. Peptide-specific T-cell lines could be generated from two of the four peptides that also proliferated in response to PAP protein stimulation. The ability of these two peptides to elicit PAP-specific Th responses suggests that they represent naturally processed PAP-specific MHC class II epitopes. There-

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<sup>3</sup> The abbreviations used are: Th, helper T cell; PAP, prostatic acid phosphatase; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; SI, stimulation index.

fore, these peptides are reasonable candidates for consideration for inclusion in peptide-based vaccines designed to elicit PAP-specific CD4<sup>+</sup> T-cell responses.

## MATERIALS AND METHODS

**Subjects.** With informed consent, peripheral blood or leukapheresis products were obtained from male subjects (27 with prostate cancer of varying disease stages and five volunteer donors without prostate cancer) at the University of Washington Medical Center between 1997 and 1999. PBMCs were isolated by Ficoll-Paque centrifugation (Pharmacia AB, Uppsala, Sweden) and cryopreserved in liquid nitrogen.

**Peptides.** The amino acid sequence of human prostatic acid phosphatase was scanned using the software analysis package *T sites* (39). On the basis of this analysis, 10 15-18-mer oligopeptides derived from the amino acid sequence of PAP were synthesized and purified to >80% by high-performance liquid chromatography, and the identity and purity was confirmed by mass spectrum analysis (United Biochemical Research, Inc., Seattle, WA). Purified peptides were reconstituted in sterile water, filtered, and stored in aliquots at -20°C. Peptide sequences are shown in Table 1.

**Evaluation of Potential Immunogenic Epitopes by T-cell Proliferative Response.** T-cell proliferative responses to PAP protein or peptide stimulation were evaluated using a modified limiting dilution analysis designed for detecting low frequency-responding T cells (40, 41). Specifically, cryopreserved PBMCs were extensively washed and resuspended in assay medium consisting of equal parts of EHAA 120 (Biofluids, Rockville, MD) and RPMI 1640 (Life Technologies, Inc., Rockville, MD) with 10 mM L-glutamine, 2% penicillin/streptomycin, 50  $\mu$ M  $\beta$ -mercaptoethanol, and 10% human AB serum (Valley Biomedical, Winchester, VA). PBMCs ( $2 \times 10^5$ /well) were plated into 96-well round-bottomed microtiter plates (Costar, Cambridge, MA) and cultured with highly purified 2.0  $\mu$ g/ml PAP (Research Diagnostics Inc., Flanders, NJ; Chemicon International, Temecula, CA), 50  $\mu$ g/ml of peptide, 2.5  $\mu$ g/ml PHA (nonspecific T-cell mitogen; positive control), or no antigen in 24-well replicate cultures for each antigen tested. Cultures were incubated at 37°C in an atmosphere of 5% CO<sub>2</sub> for 5 days. Eight h before termination of culture, cells were pulsed with 1  $\mu$ Ci of [<sup>3</sup>H]thymidine. The cultures were then harvested onto glass fiber filters, and the incorporated radioactivity was counted. The use of 24-well replicates, rather than standard 3-well replicates, permitted analysis by two different means. First, a standard SI was determined, defined as the mean of the response of the antigen-stimulated cells divided by the mean of the response of cells cultured without antigen. The second method of analysis involved scoring individual wells as positive if the [<sup>3</sup>H]thymidine uptake was greater than the mean and three SDs of the no-antigen control wells (40, 41). In this analysis, positive low-frequency events were defined if there was a significant difference at a 95% confidence level between the number of positive wells of the antigen-containing wells compared with that of the control, nonantigen containing wells. Operationally, because none of the no-antigen wells scored positive for any of the assays,  $\geq 3$  of 24 antigen-containing wells was defined as a positive event ( $P \leq 0.04$ ; one-sided Student's *t* test).

**Generation and Evaluation of Peptide-specific T-cell Lines.** PBMCs from 12 patients with prostate cancer and with no detectable PAP protein- or peptide-specific T-cell responses were resuspended at  $2 \times 10^6$  cells/ml in T-cell medium [RPMI 1640 (Life Technologies, Inc.) with 10 mM L-glutamine,

2% penicillin/streptomycin, 50  $\mu$ M  $\beta$ -mercaptoethanol, and 10% human AB serum (Valley Biomedical)] with peptide at a 10  $\mu$ g/ml final concentration. Cells were cultured in 6-well sterile plates (Costar) with  $15 \times 10^6$  starting cells. Proliferating lymphocytes were restimulated every 7–10 days with irradiated (3300 cGy) autologous PBMCs and peptide at 10  $\mu$ g/ml. Media was exchanged every 3–4 days after stimulation with T-cell medium containing 10 units/ml recombinant human IL-2 (Chiron, Emeryville, CA). After three *in vitro* stimulations, T cells in lines were found to be predominantly CD4-expressing (50–94%); however, lines were not routinely assessed for phenotype. After three to five *in vitro* stimulations, T-cell cultures were assessed for cell proliferation in response to antigen stimulation. Cultured cells ( $5 \times 10^4$ /well) were combined with  $1.5 \times 10^5$  cells/well irradiated (3300 cGy) autologous PBMCs as antigen-presenting cells in assay medium. As before, cells were stimulated with 2.0  $\mu$ g/ml PAP, 50  $\mu$ g/ml of peptide, 2.5  $\mu$ g/ml PHA, or no antigen in 4- to 8-well replicate cultures for each antigen tested. Cultures were incubated at 37°C in an atmosphere of 5% CO<sub>2</sub> for 5 days, pulsed with 1  $\mu$ Ci of [<sup>3</sup>H]thymidine, harvested, and counted as described above. Results are reported as the mean and SD in cpm of [<sup>3</sup>H]thymidine uptake. Antigen-specific proliferation was defined as significant [<sup>3</sup>H]thymidine incorporation at a 95% confidence (with  $P \leq 0.05$ ; Student's *t* test) in the antigen-stimulated wells compared with the no-antigen control wells.

## RESULTS

**Potential PAP-specific MHC Class II T-cell Epitopes Can Be Predicted by Peptide Motif Analysis.** Early studies (42, 43) identified certain protein secondary structural motifs as common to MHC class II T-cell epitopes, typically amphipathic  $\alpha$  helices. Consequently, to identify potential MHC class II epitopes for PAP, the amino acid sequence of PAP was analyzed using the software analysis package *T sites* (39). This program identifies sequence motifs common to MHC class II epitopes according to the algorithm of Rothbard and Taylor (44), as well as regions likely to form amphipathic  $\alpha$  helices, as potential T-cell recognition sites (45, 46). Results from this analysis are shown in Fig. 1. Ten potential T-cell antigenic regions were empirically chosen for study based on the prediction by both methods, as depicted by the brackets in Fig. 1 and as shown in Table 1. Unlike MHC class I molecules, which typically bind peptides of 9 to 10 amino acids in length, MHC class II molecules are generally believed to be more permissive in the length and exact amino acid sequence of bound peptide (47). In studies defining the Th epitopes of tetanus toxoid, panels of overlapping peptides were screened for their ability to stimulate T-cell proliferation in PBMCs from tetanus-immune subjects. Peptides of greater than 12 amino acids and less than 31 amino acids and typically in the range of 14 to 16 amino acids were found to be most efficient in defining recognized epitopes (48). For that reason, 15 to 18 amino-acid peptides were constructed for the current study.

**T-cell Proliferative Responses to PAP-derived Peptides Can Be Detected in Patients with Proliferative T-cell Responses Specific for PAP.** To determine whether the peptides chosen could represent PAP-specific Th epitopes, the peptides were used as stimulator antigens in proliferation assays in male subjects with and without PAP-specific T-cell responses. We have found previously (15) that some men with prostate cancer have detectable T-cell proliferative responses specific for PAP, and although these responses are generally low-level (SI < 4), the prevalence of responses is higher in patients with prostate cancer than in controls. Similar methods have been used previously (49, 50) in other systems to identify helper epitopes from large panels of overlapping peptides using PBMCs from antigen-immunized patients. Fig. 2 shows an example in which T cells obtained preoperatively from a 63-year-old male with clinically localized prostate cancer were found to proliferate in response to stimulation with PAP, with a SI of 3.3. In this subject, T-cell proliferation in response to individual peptide stimulation with a SI greater than 2.0

Table 1 PAP-derived peptides

Sequence of the 10 15–18-mer oligopeptides derived from the amino acid sequence of PAP.

Peptide	Amino acids	Sequence
p45	45–59	GDRSPIDTFPTDPIK
p81	81–95	LGEYIRKRYRKFLNE
p110	110–124	DRILMSAMTNLAALF
p184	184–198	YKDFIATLGKLSGLH
p199	199–213	GQDLFGIWSKVYDPL
p228	228–242	TEDMTKRLRELSLS
p260	260–277	GGVLVNEILNHNKRAQI
p298	298–312	MALDVYNGLLPPYAS
p308	308–322	PPYASCHLTLYFEK
p352	352–366	CPLERFAELVGPVIP

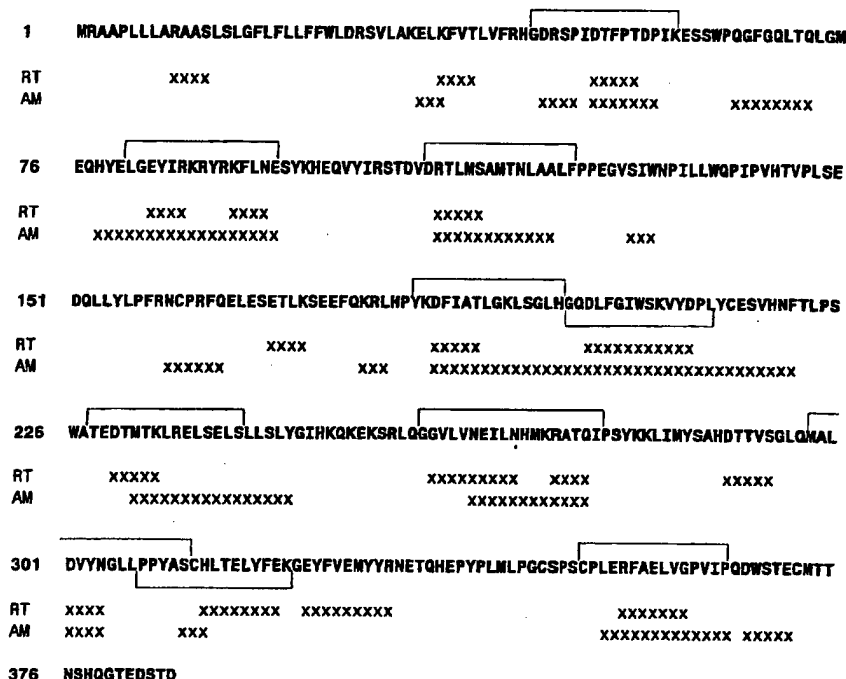


Fig. 1. T sites analysis of amino acid sequence of human PAP, and selection of potential T-cell epitopes. Shown in the top line is the amino acid sequence of human PAP. X, the residues predicted to represent T-cell epitopes based on motif algorithms of Rothbard and Taylor (RT) or predicted amphipathic  $\alpha$  helical structure (AM). Ten 15-18-mer oligopeptides were constructed based on the predictions of both methods, denoted in the brackets.

was not detected; however, responses with 3 of 24 and 4 of 24 positive wells were detected to peptides p228-242 and p199-213, respectively. This type of analysis was applied to PBMCs from 20 subjects, as shown in Fig. 3. Thirteen of these subjects had no detectable immunity to PAP (eight of which had prostate cancer and five of which were male control blood donors without prostate cancer), and seven were patients with prostate cancer with detectable immunity to PAP. In Fig. 3A, few peptide-specific responses could be detected in PBMCs from the 13 subjects who had no detectable T-cell responses specific for PAP, both patients with prostate cancer and male control donors. In Fig. 3B, however, some level of peptide-specific T-cell immunity could be detected to each of the peptides using PBMCs

from the seven prostate cancer subjects with detectable T-cell responses specific for PAP.

**p199-213 and p228-242 Represent Th Epitopes of PAP.** We next wanted to determine whether any of the peptides constructed could represent naturally processed PAP-derived helper epitopes, capable of priming T-cell responses *in vitro* to respond to PAP protein. Given that the experiments were done irrespective of binding to specific MHC class II types, we chose to prioritize for study only those peptides identified above to which responses occurred in the majority of subjects with PAP-specific T-cell responses compared with subjects without PAP-specific T-cell responses. Consequently, four peptides were chosen, p81-95, p199-213, p228-242, and p308-322, given that at least 4 of 7 of the PAP-specific PBMC specimens proliferated in response to these peptides (Fig. 3). In addition, because our ultimate goal was to identify peptides that might be capable of eliciting Th responses to PAP in patients with prostate cancer, PBMCs from 12 patients with advanced stage prostate cancer and without PAP-specific proliferative responses (data not shown) and of presumably diverse MHC class II backgrounds were chosen for limited T-cell culture experiments. PBMCs from these 12 patients were stimulated *in vitro* with each of the four peptides (p81, p199, p228, and p308). After three to five *in vitro* stimulations with peptide, T-cell cultures were assessed for proliferation in response to either the stimulating peptide or PAP protein. Fig. 4 shows an example of results from one individual patient. Before culture (Fig. 4A), this patient did not have evidence of PAP-specific or peptide-specific T-cell proliferation. After four *in vitro* stimulations with either p199-213 peptide (Fig. 4B) or p308-322 peptide (Fig. 4C), peptide-specific T-cell proliferation could be detected. No specific proliferation could be detected after culture with p81-95 or p228-242 in this particular patient (data not shown). In addition, whereas no PAP-specific T-cell proliferation could be detected in the p308-322 culture (Fig. 4C), PAP-specific T-cell proliferation could be detected in PBMCs cultured with p199-213 peptide (Fig. 4B). A summary of similar results for the 12 patients is shown in Table 2. As demonstrated, peptide-specific lines could be generated to each of the peptides in at least 1 of the 12 patient samples. Of the

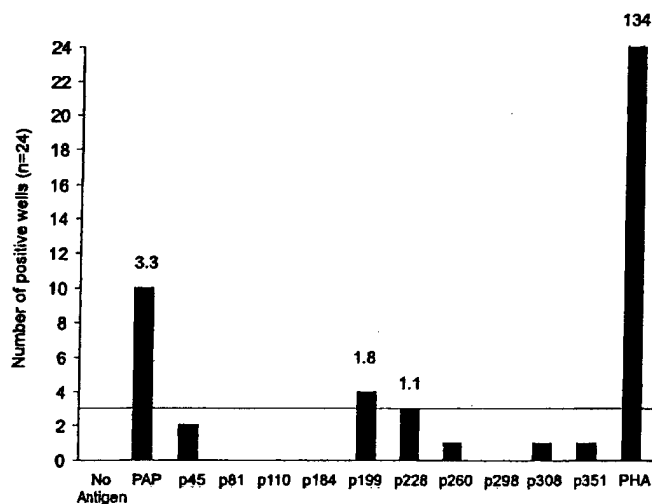
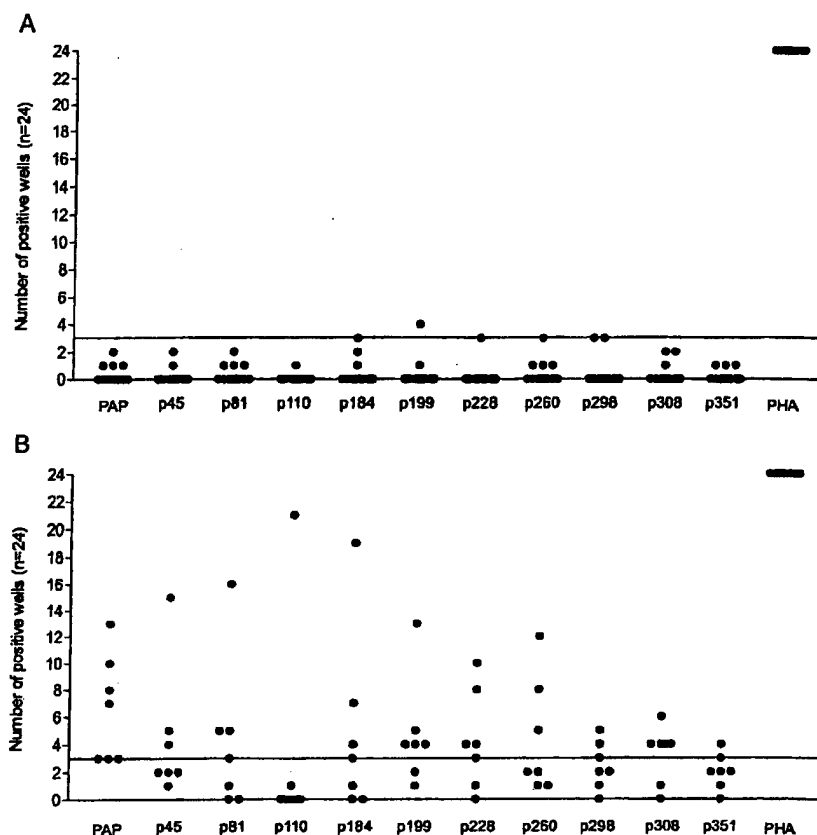


Fig. 2. T-cell proliferative responses to PAP-derived peptides can be detected. This is an example of a modified dilution proliferative T-cell assay using PBMCs from a 63-year old with early stage prostate cancer. Responses to individual peptides were tested in 24-well replicates, and individual wells were scored positive if the cpm were greater than the mean and three SD of the 24 no-antigen control wells. Positive responses were defined as  $\geq 3$  of 24 wells, indicated by the line. Numbers above the columns represent the calculated SI of the 24-well replicates.



Fig. 3. T-cell proliferative responses to PAP-derived peptides can be detected in patients with prostate cancer who have proliferative T-cell responses specific for PAP. PBMCs from 20 males with and without prostate cancer were assessed for proliferative T-cell responses to PAP, PHA, and the 10 peptides in 24-well replicates. The number of individual wells scoring positive is shown, with positive responses defined as  $\geq 3$  of 24 wells, indicated by the line. Results are shown for (A) those subjects with  $< 3$  of 24 wells specific for PAP ( $n = 13$ ) and (B) those subjects with  $\geq 3$  of 24 wells positive for PAP ( $n = 7$ ).



peptide-specific lines, however, only p199- and p228-specific lines also proliferated in response to PAP protein, suggesting that these two peptides represent naturally processed PAP-specific MHC class II epitopes.

## DISCUSSION

Th cells are of central importance in the generation of immune responses (3). Animal models have underscored the necessity of

tumor-specific Th cells directly or indirectly by recruiting other effector cells for maintenance of therapeutic antitumor immune responses (10, 51, 52). CTLs, in particular, have been shown to require CD4<sup>+</sup> Th cells for long-term memory, survival, and cytolytic efficacy (7, 8). In the current study, we used PBMCs from patients with detectable Th immunity to PAP to identify potential PAP-specific Th peptide epitopes. We report that epitopes can be identified by motif analysis and that peptides identified in this fashion could be used to prime PAP-specific Th responses *in vitro*. By this means, we identify

Fig. 4. PAP-specific T-cell lines can be established after repetitive *in vitro* stimulation with PAP-derived peptides. As an example, PBMCs obtained from a patient with prostate cancer were assessed before culture (A) and after four *in vitro* stimulations with either p199-213 peptide (B) or p308-322 peptide (C). Shown are the mean and SD of cpm of [<sup>3</sup>H]thymidine incorporation of cultures after antigen stimulation with peptide, PAP protein, PHA, or no antigen.

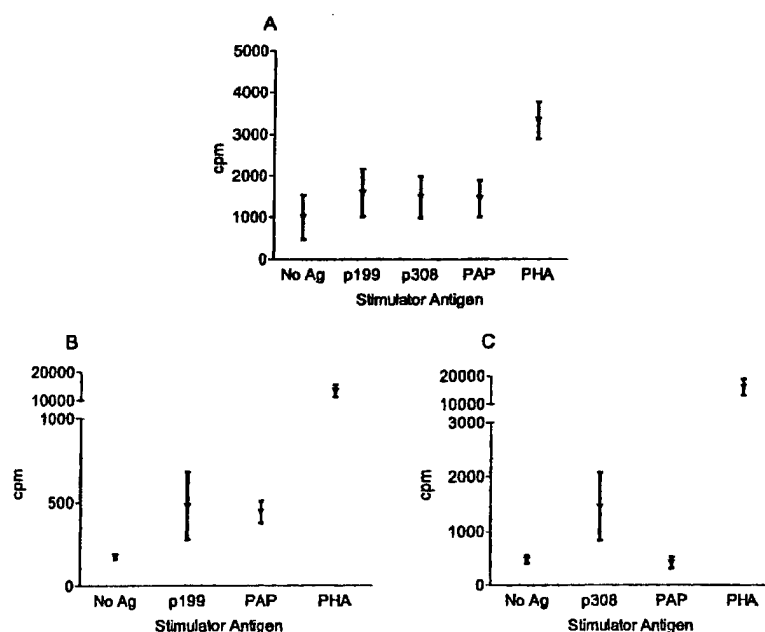


Table 2 T-cell lines specific for p199 and p228 proliferate in response to PAP protein

Shown is a summary of results for PBMCs from 12 patients with prostate cancer cultured with each of the four peptides. The number of PBMC specimens that demonstrated peptide specificity, or peptide and PAP protein specificity, after 3–5 *in vitro* stimulations with peptide are shown.

PAP-derived Th peptide	Peptide-specific	Peptide- and PAP-specific
p81	2/12	0/12
p199	4/12	3/12
p228	1/12	1/12
p308	5/12	0/12

two peptide epitopes, p199-213 and p228-242, that are capable of eliciting Th responses to PAP protein *in vitro* and, thus, likely represent naturally processed MHC class II epitopes.

The identification of Th epitopes has traditionally been done either by eluting peptides bound to specific MHC class II molecules or by screening panels of overlapping peptides derived from the antigen being studied. The first method, eluting and sequencing peptides bound to specific MHC class II molecules from antigen-presenting cells, has been used to identify MHC class II epitopes associated with insulin-dependent diabetes (53, 54), multiple sclerosis, and rheumatoid arthritis (55), as well as from the melanoma tumor antigens tyrosinase (12) and gp100 (13). Obviously, elution of peptides from specific MHC class II molecules requires selection of specific MHC restriction elements for study. This method is also potentially cumbersome, identifying epitopes from multiple processed antigens and not specifically a single antigen under study (56). Moreover, it is generally believed that MHC class II epitopes are less length- and sequence-constrained compared with MHC class I epitopes and, in fact, that specific Th epitopes may bind multiple MHC class II molecules (53, 56, 57). Because we wanted to identify epitopes that might be common to many patients of diverse MHC backgrounds, we chose to screen peptides using PBMCs irrespective of specific MHC class II types. In that regard, studies identifying Th epitopes of the diphtheria toxin identified epitopes that were common to several patients of diverse MHC class II backgrounds (50).

Other groups have identified Th epitopes by screening panels of overlapping peptides derived from the antigen being studied using PBMCs from immune individuals. Th epitopes from tetanus toxoid (48) and diphtheria toxin (50), as well as the MAGE-3 melanoma tumor antigen (14), have been identified by this method. In the case of tetanus and diphtheria toxins, panels of peptides of varying lengths were used, spanning the entire length of the proteins, as stimulator antigens in T-cell proliferation assays to determine which peptides stimulated proliferation using PBMCs from tetanus-immune individuals (48, 49). The advantage to this type of approach is that the results are exhaustive, spanning the entire length of the immunogen, and have identified peptide epitopes common to most immune patients of presumably diverse MHC class II types. In addition, this method identified peptides of 14–16 amino acids in length as efficient in eliciting Th proliferation. We adopted a similar strategy to define Th epitopes from PAP, given that we had identified previously (15) that some patients with prostate cancer have detectable Th immune responses to PAP. A comprehensive study of overlapping peptides would have been less feasible in our system, however, given that Th responses to PAP were found previously to be rare among patients and are generally of low magnitude. Consequently, an exhaustive search of overlapping peptides would have necessitated extremely large numbers of PBMCs ( $>10^9$  cells) to detect low-level responses. Therefore, we purposefully chose to bias our selection of peptides, using algorithms to identify potentially antigenic regions of the PAP protein of 15–18 amino acids in length. Although this method may certainly have missed potential epitopes, the fact that we identified two Th

epitopes of 10 peptides studied demonstrates the feasibility and utility of this method.

In the current study, we identified peptides derived from PAP, Th proliferative responses to which were associated with responses to soluble PAP protein. In fact, responses to each of the peptides were found in at least one of the seven patients with previously identified Th responses to PAP. This suggests that all of these peptides could represent PAP Th epitopes presented by different MHC class II epitopes present in some individuals but not in others. We were particularly interested in identifying epitopes that might be capable of binding multiple MHC class II types and, therefore, be more "universal" as Th epitopes, similar to peptides identified for diphtheria toxin (50). Consequently, we prioritized for study only those four peptides identified that stimulated T-cell proliferation in the majority of PBMC samples that also exhibited PAP protein-specific T-cell proliferation. By repetitive *in vitro* stimulation, peptide-specific T-cell lines could be generated from each of these four peptides from at least 1 of 12 PBMC specimens obtained from patients with prostate cancer who had no detectable preexisting peptide-specific or PAP-specific Th responses. Peptide-specific lines derived from two peptides, p199-213 and p228-242, were also able to proliferate in response to PAP protein stimulation, suggesting that these represent MHC class II epitopes naturally presented after antigen processing.

The development of vaccines capable of eliciting prostate-specific immunity for the treatment of prostate cancer is an active area of research (20). Clinical trials underway include cytokine-transfected whole cell vaccines (58), protein-based vaccines (21), viral-based vaccines (59, 60), and dendritic cell vaccines pulsed with either MHC class I-restricted peptide epitopes (61) or proteins, including PAP (22, 62). Immunization directly with MHC class I peptides represents another vaccination approach that has shown success in eliciting antigen-specific CD8<sup>+</sup> T cells in human clinical trials, in some cases with the suggestion of clinical benefit (63–65). Similar trials (16, 23–27) have been contemplated in prostate cancer, particularly because MHC class I epitopes have already been identified for PAP, prostate-specific antigen, and prostate-specific membrane antigen. Animal studies (19) have suggested the importance of CD8<sup>+</sup> T-cell immunity for actual immune-mediated destruction of prostate tissue. Consequently, antigen-specific Th cells, and Th1 cells in particular, may be particularly important for the effectiveness and maintenance of prostate-specific CTLs (9, 15, 66). Data presented here represents the first report of PAP-specific Th epitopes and a demonstration that PAP-specific Th responses can be elicited *in vitro* after culture with these epitopes. Ongoing studies will evaluate whether peptide-specific lines exhibit a Th1- or Th2-type bias with respect to cytokine secretion. Given the importance of Th cells in effective antitumor immune responses, vaccine strategies, and MHC class I peptide-based vaccine strategies in particular, targeting this protein might be further enhanced by inclusion of these epitopes capable of inducing PAP-specific Th responses.

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## Naturally Occurring Prostate Cancer Antigen-Specific T Cell Responses of a Th1 Phenotype Can Be Detected in Patients With Prostate Cancer

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**BACKGROUND.** Cytotoxic T cells (CTL) are considered one of the primary effector cell populations in antitumor immunity. Recent studies, however, have demonstrated the critical importance of helper T cells (Th), specifically interferon  $\gamma$  (IFN $\gamma$ )-secreting Th1 cells, either by supporting an appropriate CTL environment or by recruiting other effector cells. We evaluated whether patients with prostate cancer have naturally occurring Th-cell responses specific for two prostate cancer-associated antigens, prostate-specific antigen (PSA) and prostatic acid phosphatase (PAP), and whether Th1-type responses to these antigens could be detected.

**METHODS.** Peripheral blood mononuclear cells (PBMC) were collected from 80 patients with prostate cancer and 20 male controls without prostate disease. Th-cell responses were evaluated by measuring antigen-specific proliferation. IFN $\gamma$  and IL-5 secretion in response to antigen stimulation was determined by enzyme-linked immunosorbent assay.

**RESULTS.** T cell proliferative responses specific for PSA and PAP could be detected in patients with prostate cancer. Six percent (5/80) of patients had T cell responses specific for PSA and 11% (9/80) for PAP. T cell responses specific for PSA were more prevalent in patients with metastatic disease ( $P = 0.02$ ), whereas responses specific for PAP could be detected in patients irrespective of disease stage. IFN $\gamma$ -producing Th cells, specific for both PSA and PAP, could be identified in patients with prostate cancer.

**CONCLUSIONS.** Patients with prostate cancer can have detectable Th-cell responses specific for the prostate cancer-associated proteins PSA and PAP. The presence of antigen-specific Th1 immune responses in prostate cancer patients suggests that an immune environment capable of supporting antigen-specific CTL may exist in vivo. *Prostate* 47:222–229, 2001.

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**KEY WORDS:** prostate cancer; helper T cells; prostate-specific antigen; interferon-gamma; IL-5; Th1 cells

Abbreviations: CTL, cytotoxic T lymphocyte; DTH, delayed-type hypersensitivity; ELISA, enzyme-linked immunosorbent assay; IFN $\gamma$ , interferon gamma; IL-5, interleukin 5; OD, optical density; PAP, prostatic acid phosphatase; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; PSA, prostate-specific antigen; SD, standard deviation; S.I., stimulation index; Th, helper T cell; TMB, tetramethylbenzidine; TNF  $\alpha$ , tumor necrosis factor  $\alpha$ .

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## INTRODUCTION

Several cell populations are involved as effector cells in antitumor immune responses, including lymphocytes, natural killer cells, macrophages, and eosinophils. Cytotoxic T lymphocytes (CTL) have long been considered to be the primary effector cell population critical for effective antitumor immunity, given their ability to discriminate and lyse cells expressing tumor-associated antigenic peptides in the context of MHC class I molecules [1-3]. Rodent models of experimental autoimmune prostatitis have suggested that CTLs are critical for actual destruction of prostate tissue [4-6]. These findings have suggested that the induction of tumor-specific CTL will likely be important for the immunological treatment of prostate cancer [7,8].

Recent studies have demonstrated the central involvement of helper T cells (Th) in antitumor immunity [9-12]. Th cells may participate by supporting the development of effective CTL [13-15], by themselves lysing tumor cells independent of CD8+ CTL [16], or potentially by recruiting other effector cells, including macrophages and eosinophils [9,10,17]. In general, Th cell responses tend to be polarized to either a Th1-like or Th2-like phenotype based on the patterns of cytokines secreted [18]. The Th1 phenotype, typified by secretion of lymphotoxin, tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) and interferon  $\gamma$  (IFN $\gamma$ ), has been more associated with cellular inflammatory immune responses with concurrent antigen-specific CTL, activated macrophages, and delayed-type hypersensitivity (DTH) reactions. This type of immune response has been demonstrated to be protective against the development of certain types of intracellular pathogens, in mediating various tissue destructive autoimmune states, and in eliciting antitumor responses [10,19-21]. By contrast, the Th2 immune response, typified by secretion of the interleukins IL-4, IL-5, IL-6, IL-10, and IL-13, has been more associated with promoting humoral immune responses and the development of peripheral tolerance [19,22-24].

Studies in patients with metastatic prostate cancer have suggested that T cells nonspecifically stimulated by mitogenic stimuli tend to be Th2 biased, as compared with normal donors [25-27]. These types of findings have led investigators to speculate that an imbalance of Th subsets may lead to decreased immune surveillance and tumor escape in patients with cancer [28]. Others have suggested that impaired signal transduction in peripheral T cells in patients with prostate cancer may lead to decreased tumor surveillance [29]. In this report, we explored whether patients with prostate cancer have Th responses specific for two prostate cancer-associated antigens,

prostate-specific antigen (PSA) and prostatic acid phosphatase (PAP), by evaluating T cell proliferation in response to antigen stimulation. In addition, we determined whether Th1 responses to these antigens could be detected in patients with prostate cancer, implying an immune environment capable of supporting antigen-specific CTL.

## METHODS

### Subject Populations

With informed consent, 70 ml blood were obtained from 80 patients with prostate cancer, as well as 20 male volunteer blood donors without histories of prostate disease, at the University of Washington Medical Center between 1997 and 1999. Peripheral blood mononuclear cells (PBMC) were prepared by Ficoll-Paque centrifugation (Pharmacia AB, Uppsala, Sweden) and cryopreserved in liquid nitrogen. PBMC were classified according to the patient's disease stage and treatment status into two general groups, each with 40 patients. The first group consisted of patients with clinically localized prostate cancer, samples collected either before treatment or at a variety of times following primary therapy. The second group consisted of patients with stage D prostate cancer, either responsive or unresponsive to androgen ablative therapy.

### Detection of Antigen-Specific T Cell Immunity by $^3\text{H}$ -Thymidine Incorporation Assay

Cryopreserved PBMCs were extensively washed and resuspended in media consisting of equal parts of EHAA 120 (Biofluids, Rockville, MD) and RPMI 1640 (Gibco BRL, Rockville, MD) with 10 mM L-glutamine, 2% penicillin/streptomycin, 50  $\mu\text{M}$   $\beta$ -mercaptoethanol, and 10% human AB serum (Valley Biomedical, Winchester, VA). Cells were then analyzed for a T cell proliferative response following exposure to 0.5-2.0  $\mu\text{g}/\text{ml}$  PAP (Chemicon Int., Temecula, CA; Research Diagnostics, Inc. (RDI), Flanders, NJ), 0.5-2.0  $\mu\text{g}/\text{ml}$  PSA (Chemicon; RDI), or 2.0  $\mu\text{g}/\text{ml}$  ovalbumin (Sigma Chemical Co., St. Louis, MO), as a negative control protein. Phytohemagglutinin (PHA), a nonspecific T cell mitogen, was used at 2.5  $\mu\text{g}/\text{ml}$  as a positive control.  $2 \times 10^5$  PBMC/well were plated into 96-well round bottom microtiter plates (Costar, Cambridge, MA) in 12-well replicate cultures for each concentration of protein tested and incubated at 37°C in an atmosphere of 5% CO<sub>2</sub> for 5 days. Eight hours before termination of culture, cells were pulsed with 1  $\mu\text{Ci}$   $^3\text{H}$ -thymidine (New England Nuclear, Wilmington, DE). The cultures were then harvested onto glass fiber filters and the incorporated radioactivity was counted.

The results are reported as a stimulation index (S.I.), defined as the mean of experimental wells divided by the mean of the control wells (no antigen). For all of the assays performed, the same three lots of purified protein were used to ensure reproducibility of results. All positive results were confirmed with two different lots of protein, with the S.I. reported as the mean of the two assays. A result was considered significant if the S.I. was greater than the mean +3 SD of the S.I. determined for the control population ( $n=20$ ). All patients had proliferative responses to PHA, defined as an S.I.  $>3$ .

#### Characterization of the T Cell Response by Cytokine Release

T cells proliferating in response to antigen stimulation were characterized with respect to phenotype, Th1 or Th2, by cytokine release quantified by enzyme-linked immunosorbent assay (ELISA). Short-term T cell cultures were established from PBMC as above with 2  $\mu\text{g/ml}$  PSA, 2  $\mu\text{g/ml}$  PAP, 2.5  $\mu\text{g/ml}$  PHA, or no antigen for 5 days at 37°C/5%  $\text{CO}_2$ . Murine monoclonal antibodies specific for either human IFN $\gamma$  (Genzyme, Cambridge, MA) or human IL-5 (Pharmingen, San Diego, CA) were diluted to 2.5  $\mu\text{g/ml}$  in 50 mM sodium carbonate buffer (pH 9.6) and adsorbed to wells of Immulon-4 polystyrene plates (Dynex Technologies Inc., Chantilly, VA) overnight at 4°C. Wells were then blocked with PBS/1% BSA/0.05% Tween-20 for 3 hr at room temperature, and then washed with PBS/0.1% Tween-20. Supernatants from the short-term cultures were then pooled, and 50  $\mu\text{l}$  were added to replicate experimental wells. A standard curve of either purified IFN $\gamma$  (RDI) (0.24–4,000 pg/ml) or IL-5 (Pharmingen, San Diego, CA) (0.24–4,000 pg/ml) was included on each ELISA each plate. Plates were then incubated overnight at 4°C, washed, then incubated for 2 hr at room temperature with a biotinylated secondary antibody (anti-IFN $\gamma$ , Pharmingen; anti-IL5, Pharmingen) diluted to 1  $\mu\text{g/ml}$ . After washing, the plates were incubated for 1 hr at room temperature with peroxidase-conjugated streptavidin (Amersham, Arlington Heights, IL) diluted 1:3,000, washed again, and developed with TMB peroxidase substrate (Kierkegard and Perry Laboratories, Gaithersburg, MD). Reactions were stopped with addition of 0.5 N HCl and the optical densities (ODs) of experimental wells were read at 450 nm. Concentrations of IFN $\gamma$  or IL-5 were calculated by comparison of the obtained OD with the standard curve. Antigen-specific concentrations were determined by subtracting the background concentrations determined from the "no-antigen" control wells. Data reported show the mean and standard deviation of 4-well replicates for each culture sample.

For IFN $\gamma$  the limit of detection was 8 pg/ml, and for IL-5 the limit of detection was 2 pg/ml.

#### Statistical Analysis

Comparison of study populations was performed using a  $\chi^2$  test, with  $P \leq 0.05$  considered statistically significant.

## RESULTS

### Patients With Prostate Cancer Can Have Detectable T Cell Proliferative Responses Specific for Two Prostate Cancer-Associated Proteins, PSA and PAP

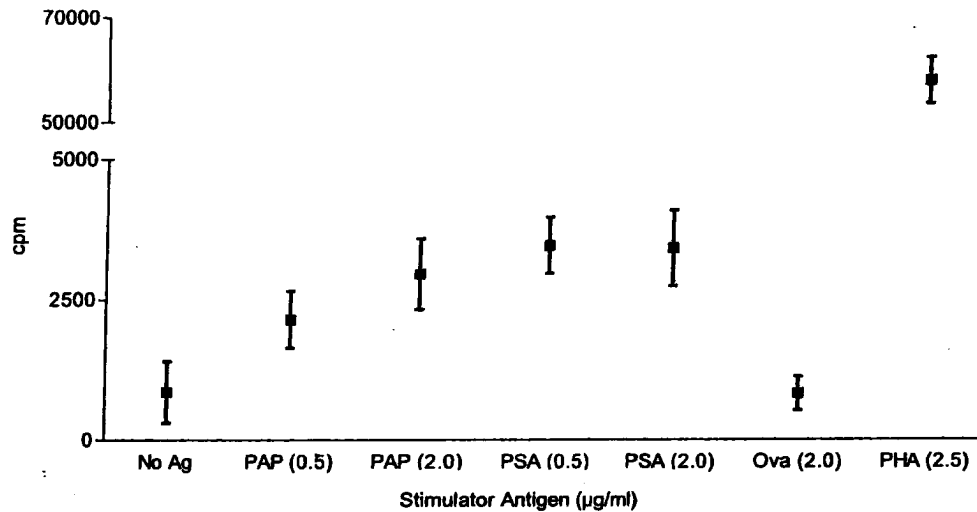
T cell proliferative responses specific for PSA and PAP were detected in patients with prostate cancer. As an example, PBMC obtained from a 51-year-old male with stage D1 prostate cancer were stimulated in vitro with varying concentrations of PAP, PSA, ovalbumin or PHA (Fig. 1). In this patient, significant T cell proliferation was detected in response to PAP (S.I. 3.4 at 2  $\mu\text{g/ml}$  PAP concentration) and PSA (S.I. 3.9 at 2  $\mu\text{g/ml}$  PSA concentration) stimulation. Response to PAP was found to be dose-responsive in several patients (data not shown), with significant differences detected at a concentration of 2  $\mu\text{g/ml}$ . For this reason, subsequent analysis was conducted at a standardized protein concentration of 2  $\mu\text{g/ml}$ .

### Patients With Metastatic Prostate Cancer Can Have T Cell Proliferative Responses Specific for PSA

Eighty patients with various stages of prostate cancer and 20 controls were evaluated for T cell proliferation in response to PSA stimulation. As shown in Figure 2, patients with prostate cancer could be detected with significant recall responses to PSA, particularly patients with metastatic disease. The mean S.I. of the control population was found to be  $0.9 \pm 0.4$ . Five patients (12%) with metastatic prostate cancer were found to have significant T cell responses, defined as an S.I. greater than the mean +3 SD of the S.I. of the control population. This was notably different from the population of patients with limited stage disease, none of whom (0/40) was found to have an S.I. greater than 3 SD from the mean of the control population ( $P = 0.02$ ).

### Patients With Prostate Cancer Can Have T Cell Proliferative Responses Specific for PAP, Irrespective of the Stage of Disease

The same patients and controls were evaluated for T cell proliferation in response to PAP stimulation, as shown in Figure 3. Unlike PSA, no differences were



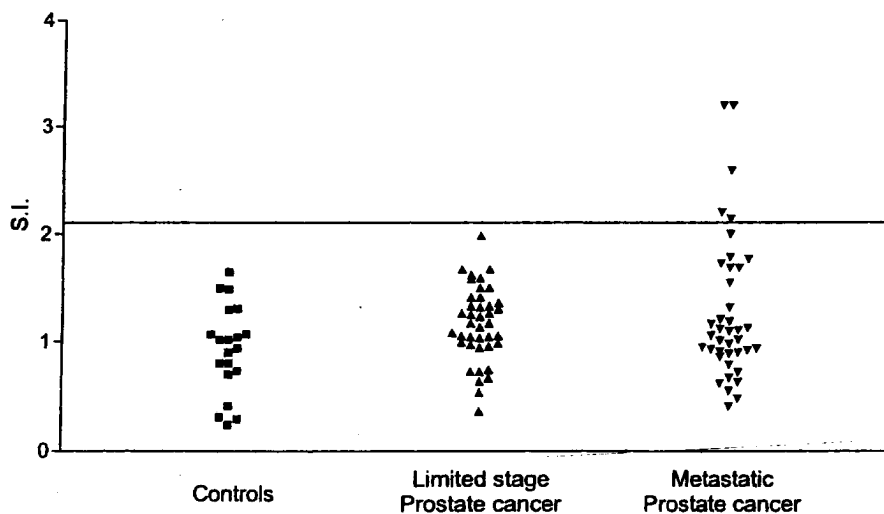
**Fig. 1.** Patients with prostate cancer can have detectable T cell proliferative responses specific for two prostate cancer-associated proteins, PSA and PAP. PBMC obtained from a 51 year old with stage D1 prostate cancer were stimulated in vitro with PAP, PSA, ovalbumin (ova), or PHA as a positive mitogenic control. Shown is the mean cpm and standard deviation of 24-well replicates.

noted with respect to stage of disease. Overall, 9/80 (11%) patients were found to have significant recall responses to PAP, defined as an S.I. greater than the mean + 3 SD of the S.I. of the control population. In addition, PBMC from three of these nine patients also had significant proliferative T cell responses to PSA.

#### T Cell Proliferative Responses Specific for PAP and PSA of the Th1 Phenotype Can Be Detected in Patients with Prostate Cancer

Figure 4 shows a representative quantitative ELISA for a 52-year-old (patient ID 3132) with androgen-

dependent metastatic prostate cancer who had been found to have a low-level proliferative T cell response to both PAP (S.I. 2.8) and PSA (S.I. 2.1). The supernatants from 96-hr cultures were found to contain 126 pg/ml IFN $\gamma$  in response to PAP, and 15 pg/ml IFN $\gamma$  in response to PSA stimulation. No IL-5, a surrogate for a Th2-type response, could be detected, and no IFN $\gamma$  could be detected in the absence of PAP or PSA. PHA stimulation resulted in both IFN $\gamma$  and IL-5 release. Of the 11 patients above with S.I. in response to either PSA or PAP stimulation greater than 3 SD from the mean S.I. of the control population, nine could be evaluated for evidence of cytokine production. A



**Fig. 2.** Patients with metastatic prostate cancer can have T cell responses specific for PSA. PBMC from 40 patients with limited stage prostate cancer, 40 patients with metastatic prostate cancer, and 20 male controls were assessed for proliferative T cell recall responses to PSA. The dots represent the S.I. obtained for individual subjects. The line represents 3 SD from the mean (S.I. =  $0.9 \pm 0.4$ ) of the S.I. for the control population (S.I. = 2.1).



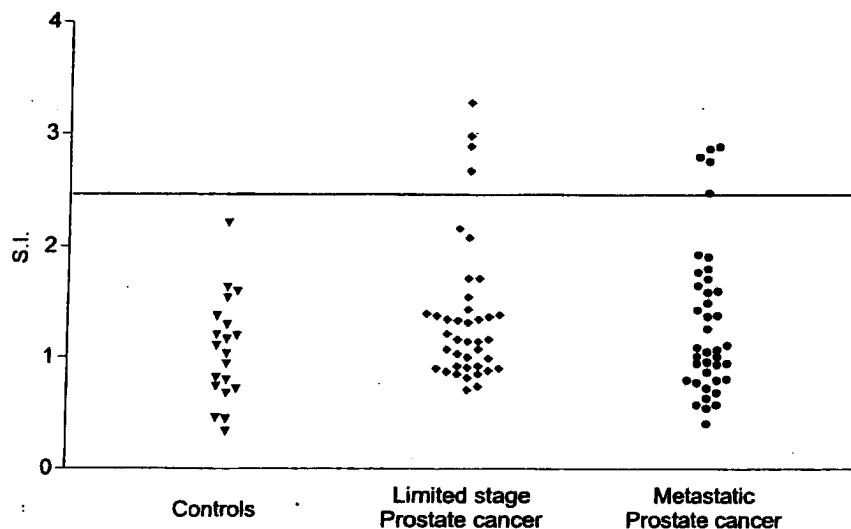


Fig. 3. Patients with prostate cancer can have T cell proliferative responses specific for PAP, irrespective of the stage of disease. PBMC obtained from the same patients were assessed for proliferative T cell recall responses specific for PAP. The dots represent the S.I. obtained for individual subjects. The line represents 3 SD from the mean ( $S.I. = 1.1 \pm 0.4$ ) of the S.I.s for the control population ( $S.I. = 2.3$ ).

summary of these results is shown in Table I. Of these nine patients, none had measurable quantities of IL-5 secretion in response to PSA or PAP stimulation, although IL-5 secretion was detected in response to PHA stimulation, the positive control. IFN $\gamma$  secretion was detected in two patients in response to PSA stimulation, and in three patients in response to PAP stimulation. All patients had measurable levels of IFN $\gamma$  in response to PHA stimulation.

### DISCUSSION

CTLs are generally believed to be the primary immune effector cells in antitumor immunity, and specifically necessary to destroy prostate tissue in vivo. Studies in rodent experimental autoimmune prostatitis have demonstrated that adoptive transfer of prostate-specific T cells alone are capable of destroy-

ing prostate tissue [6]. In an antigen-specific model of autoimmune prostatitis, Fong and colleagues demonstrated that immunization of rats against PAP using an immunization strategy capable of eliciting PAP-specific CTL resulted in destructive prostatitis that was not seen in animals without a PAP-specific CTL response [4]. Likewise, Liu and colleagues have demonstrated that T cells specific for rat prostatic steroid-binding protein are also capable of inducing destructive autoimmune prostatitis [5]. Consequently, many investigators exploring vaccine approaches for the treatment of prostate cancer have focused on strategies capable of eliciting CTL [7,8,30,31].

Several lines of evidence have demonstrated that long-lived, effective CTL responses require a supporting helper T cell environment, typically of a Th1 phenotype [9,13-15,32,33]. Because PSA and PAP are proteins whose expression is essentially restricted to

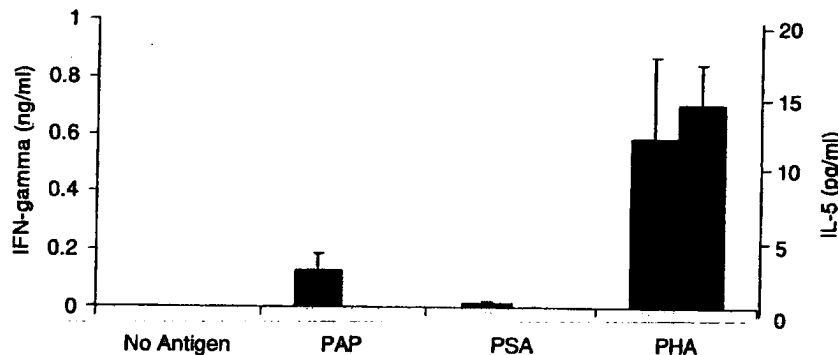


Fig. 4. T cells specific for prostate cancer antigens can secrete IFN $\gamma$ . PBMC were stimulated in the presence of PAP, PSA, PHA, or no antigen. ELISA was used to detect the production of IFN $\gamma$  or IL-5 from the culture supernatants. Black bar represents IFN $\gamma$  concentration, gray bar represents IL-5 concentration.

TABLE I: Th1-Type Proliferative Responses Can Be Detected in Patients With Prostate Cancer Specific for PAP or PSA\*

Patient ID	PAP cytokine responses			PSA cytokine responses			PHA cytokine responses		
	PAP SI	IFN $\gamma$ (pg/ml)	IL-5 (pg/ml)	PSA SI	IFN $\gamma$ (pg/ml)	IL-5 (pg/ml)	PHA SI	IFN $\gamma$ (pg/ml)	IL-5 (pg/ml)
2071	2.2	<8	<2	1.9	<8	<2	100	>2000	319 $\pm$ 38
2121	2.7	<8	<2	1.3	<8	<2	71	1897 $\pm$ 1432	237 $\pm$ 63
3046	2.5	<8	<2	3.2	<8	<2	106	>2000	149 $\pm$ 47
3048	2.8	<8	<2	3.2	<8	<2	243	>2000	450 $\pm$ 57
3089	1.6	<8	<2	2.2	<8	<2	109	1084 $\pm$ 474	35 $\pm$ 15
3132	2.8	126 $\pm$ 61	<2	2.1	15 $\pm$ 3	<2	31	582 $\pm$ 288	14 $\pm$ 3
3168	2.9	8 $\pm$ 14	<2	1.7	<8	<2	65	3017 $\pm$ 152	721 $\pm$ 78
4111	0.4	<8	<2	2.6	<8	<2	43	>2000	1415 $\pm$ 297
5024	3.0	62 $\pm$ 28	<2	1.7	95 $\pm$ 72	<2	166	>2000	178 $\pm$ 29

\*Summary of results of cytokine ELISA results for nine patients with evidence of proliferative T cell responses to PSA and/or PAP.

the prostate, we explored whether T cell responses specific for these proteins could be detected in patients with prostate cancer. We then evaluated whether IFN $\gamma$ -secreting, Th1-type cells could be detected, suggesting an immune environment capable of supporting antigen-specific CTL.

We find that preexisting T cell responses to PSA and PAP can be identified in a minority of patients with prostate cancer. The prevalence and magnitude of detectable responses to both antigens were low. This is perhaps not surprising, given that these are autologous proteins, and therefore same degree of immune tolerance to these proteins is expected. Unlike infectious disease models, in which antigens are foreign proteins and therefore usually elicit robust immune responses, identified tumor antigens are typically "self" or "altered-self" proteins [34,35]. The presence of even a low-level immune response in some patients with antigen-expressing tumors, however, suggests that immune tolerance to the antigen can be circumvented in vivo. Results from a clinical vaccine study, in which patients with prostate cancer were immunized with PSA protein, found that two of ten patients prior to vaccination had a detectable CD4<sup>+</sup> (typically Th) cell response specific for PSA. These findings are certainly consistent with our prevalence results.

In the case of PSA, responses were detected in patients with metastatic disease but not early stage disease. T cell responses specific for PAP were found in patients irrespective of disease stage. Alexander and colleagues have recently reported that proliferative T cell responses to PSA, but not PAP, could be detected in 5/14 patients with chronic prostatitis [36]. They suggest that the presence of responses to PSA in this population implies that PSA may be a natural target of an autoimmune response, and therefore a

reasonable target for immune-based treatments of prostate cancer [36]. Our results represent the first report detailing naturally occurring T cell responses specific for PSA and PAP in a large population of patients with prostate cancer. Of note, there was no history of prostatitis in the five patients identified in the current study with T cell responses specific for PSA, although a detailed history of prostatitis symptoms was not obtained from all study subjects. A priori, it is not clear why the incidence of T cell responses to PAP would be different from PSA, or why the incidence of responses would be different in distinct patient populations. Both proteins are essentially tissue-specific and secreted proteins expressed in normal and malignant prostate cells. It is possible that differences in the incidence of T cell responses is due to differences in the immunogenicity of these proteins, as has been suggested [36]. We have previously reported that patients with metastatic prostate cancer, but generally not patients with earlier stage disease, can have detectable antibody responses to PSA [37]. Those antibody responses were all of the IgG type (unpublished observations), further implicating the presence of PSA-specific Th cells involved in immunoglobulin class switch.

A small number of patients with prostate cancer could be identified with a type 1 pattern of T cell cytokine secretion in response to PSA or PAP stimulation. Previous studies evaluating cellular immune responses in patients with prostate cancer have suggested that patients have a global Th2 bias [25-27]. Elsasser-Beile and colleagues have reported that lymphocyte cultures from patients with prostate cancer stimulated by mitogens produce less IFN $\gamma$  when compared to normal controls [25] and that this tends to be most pronounced in patients with higher

tumor burdens [26]. These studies, however, were conducted to evaluate nonspecific mitogenic stimulation, and did not evaluate antigen-specific responses. Our findings demonstrate that patients with prostate cancer can, in fact, have Th1-type proliferative T cell responses to autologous prostate-specific proteins. While this does not exclude the possibility that Th2 responses to these antigens can exist, it would suggest that a tumor antigen-specific Th2 bias does not necessarily occur in patients with prostate cancer.

Clinical vaccine trials for patients with prostate cancer targeting PSA and PAP are under investigation by several groups [30,38–41]. Vaccine strategies, including dendritic-cell-based strategies, known to elicit Th1 responses and CTL responses, are of particular interest, as such strategies have been suggested to have clinical benefit [41,42]. The results reported here demonstrate that Th1 responses to prostate cancer-associated proteins can exist in vivo, and therefore may potentially be either elicited or augmented through antigen-specific vaccine strategies capable of producing this type of immunity. Results from ongoing and future prostate cancer vaccine trials should determine whether these strategies are indeed capable of eliciting a prostate-destructive CTL response, and whether that response is associated with a therapeutic response.

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